Molecular Characterisation of the Arabidopsis TT12 MATE Protein and Functional Analysis of other Plant MATE Transporters

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SUMMARY

Flavonoids are a diverse group of plant secondary metabolites that accumulate in a variety of plant tissues and play many roles in important processes including stress protection, defence and seed dispersal. To accomplish many of these functions, flavonoids accumulate at high concentrations. However, the denaturing properties of these phenolic substances make them toxic to the cytosol. Therefore, flavonoids undergo secretion into the extracellular space or accumulate in the vacuole. The knowledge of transport mechanisms involved in the accumulation of flavonoids is limited, even though the biosynthetic pathway is well known. Recent reports suggest that MATE proteins are involved in flavonoid transport and act as cation H^+/Na^+-antiporters. However, the identification of conserved amino acid residues or domains essential to a transport function remains poorly characterised, with no reports for plant MATEs. TT12 is a vacuolar flavonoid/H^+-antiporter expressed exclusively in the seed coat endothelium actively involved in the transport of glucosylated anthocyanins (Marinova *et al*, 2007).

In this work we describe strategies to (i) functionally identify important amino acid residues using *TT12* as a model for plant MATE family, (ii) functionally characterise two novel MATE transporters in *Lupinus albus* implicated in citrate and isoflavonoids transport and (iii) identify novel flavonoid transporters within the 56 members of the Arabidopsis MATE family.

(i) Since *TT12* gene was well characterised in our laboratory and due to its easily scorable seed phenotype, we decided to use *TT12* as a model to investigate which amino acids are crucial for proper functioning of plant MATE transporters. Given that transport critical residues for NorM from *Vibrio parahaemolyticus* and hMATE1 from *Homo sapiens* were identified and we found an amino acid residue homologous to them in *TT12* (E290), we performed site-directed mutagenesis on E290 and nine others amino acid residues. In the range of experiments we searched specially for the mutated versions of TT12 which were unable to functionally complement the PA deficient seed phenotype of *tt12* mutant. We describe that only a single amino acid (E290) failed to complement the tt12 seed phenotype and further show that this mutated version completely lacks transport activity on cyanidin 3-glucoside (the substrate for the native TT12).

(ii) White lupins characteristically produce cluster roots under phosphate starvation. These cluster roots are known to excrete both citrate and isoflavonoids into the rhizosphere. Previous work within the group identified two full length cDNAs expressed in cluster roots. These were designated LaMATE1 and -2. We investigated if these cDNAs, when heterogously expressed in yeast, were able to transport either citrate or the isoflavonoid, genistein. We describe that

LaMATE1 was unable to transport citrate but LaMATE2 transported genistein, when uptake experiments were conducted with microsomes.

(iii) Using previously published data and *in silico* sequence analyses, we selected three candidate genes for novel flavonoid transporters.

AtDTX35 (At4g25640) has been reported to be homologous to a tomato MATE which is upregulated in the tomato mutant, ANT1 encoding proteins involved in the anthocyanin biosynthesis (Mathews *et al*, 2003). We started our approach with downregulation of *DTX35* gene with RNAi in PAP1 overexpressing line, which due to hyperaccumulation of anthocyanin exhibits purple pigmentation. Interestingly, numerous lines carrying the RNAi construct reverted from purple color to green. We describe that *DTX35* was unable to complement tt12 seed phenotype and its promoter tissue activity is not specific in young seedlings.

AtDTX31 (At1g12950) has been shown to be transcriptionally upregulated in Arabidopsis roots under salt treatment and displayed a similarity to proteins predominantly expressed in tomato fruits (Maathuis, 2006). To investigate if *DTX31* is expressed in salt stress conditions, we performed sqPCR analyses and examined if DTX31 promoter activity is also tissue specific. We describe that DTX31 is highly expressed in the roots of Arabidopsis subjected to salt stress and its promoter's spatial activity in response to high concentration of salt.

AtDTX28 (At5g44050) has been reported to be induced after UV-B treatment, which is a main reason for increase in the transcript abundance of genes, including MYB12 (the transcription factor) which are involved in the flavonol biosynthesis. We investigated if *DTX28* expression in MYB12 overexpressing line, myb12 knock-out line and triple knock-out line myb11-myb12-myb111 is different in comparison to the wild-type line by sqPCR and HPLC analyses. We describe that DTX28 does not show higher transcript level in MYB12 overexpressing lines compared to the wild-type and no differences were observed in metabolic profiles between mutants and a wild-type.

ZUSAMMENFASSUNG

Flavonoide sind Sekundärmetabolite die zur Klasse der phenolischen Substanzen gehören. Sie werden in den meisten Pflanzengeweben akkumuliert und spielen bei der Reaktion der Pflanze gegen Stresssituationen und Pflanzenpathogene sowie bei der Verbreitung von Samen eine wichtige Rolle. Um diese Funktionen auszuüben, werden Flavonoide in hohen Konzentrationen synthetisiert und akkumuliert. Da Phenole aber potentiell toxisch sind, dürfen sie nicht im Cytosol vorliegen. Deshalb werden Flavonoide entweder in den Apoplasten exkretiert oder in der Vakuole gelagert. Unser Wissen über die Transportmechanismen, die zu einer vakuolären Akkumulation der Flavonoide führen, sind immer noch limitiert obwohl deren Biosynthese sehr gut aufgeklärt ist. Neuere Untersuchungen deuten darauf hin, dass MATE Proteine am Flavonoidtransport beteiligt sind und als Kationen (H^+ oder Na^+)/Flavonoid Antiporter wirken. Unser Wissen, welche MATE Proteine tatsächlich am Flavonoidtransport beteiligt sind ist aber noch sehr gering. Hinzu kommt, dass auch noch sehr wenig darüber bekannt ist, welche konservierten Aminosäuren oder Domänen für die Transportfunktion wichtig sind; speziell für pflanzliche MATEs ist dahingehend bisher noch nichts beschrieben. TT12 ist ein vakuolärer Flavonoid/H^+ Antiporter, der ausschliesslich im Endothel der Samenschale exprimiert wird und glukosilierte Anthocyane transportiert (Marinova *et al* 2007).

In der vorliegenden Arbeiten werden Strategien beschrieben, um (i) anhand von TT12 Aminosäuren zu identifizieren, die für die Funktion von MATEs wichtig sind, (ii) zwei MATEs von *Lupinus albus* zu charakterisieren und (iii) innerhalb der Klasse der MATEs von Arabidopsis, die 56 Mitglieder enthält, neue Flavonoidtransporter zu identifizieren.

(i) Da TT12 in unserem Labor gut charakterisiert wurde und da ein Phänotyp leicht erkennbar ist (proanthcyanidinedefizient), haben wir uns entschlossen TT12 als Model zu verwenden um herauszufinden, welche Aminosäuren für die korrekte Funktion von pflanzlichen MATEs wichtig sind. Da für die zwei MATEs NorM (*Vibrio parahaemolyticus)* und hMATE1 (*Homo sapiens*) gezeigt wurde, das eine bestimmte Aminosäure, die auch in TT12 konserviert ist (E290), für die Funktion eine entscheidende Rolle spielt, haben wir diese und neun weitere Aminosäuren mutagenisiert. Nachdem die mutierten Formen in die *tt12* Mutante eingeführt wurden haben wir die Samen mit den mutierten TT12 Versionen danach untersucht, ob die mutierte Version den Phänotypen nicht mehr komplementieren kann. Wir haben gesehen, dass nur die E290 Mutation den Phänotypen ganz und gar nicht mehr komplementieren konnte und konnten mit Transportmessungen auch zeigen, dass diese Version Cyanidin 3-Glukosid (das Substrat von TT12) nicht mehr transportieren kann.

(ii) Weisslupine produziert unter phosphatlimitierenden Bedingungen charakteristische Clusterwurzeln. Es wurde beschrieben, dass diese Wurzeln Citrat und Isoflavonoide in die

Rhizosphäre exkretieren. In einer vorangegangenen Arbeit in unserem Labor wurden zwei Volllänge cDNAs von MATE Proteinen aus Clusterwurzeln identifiziert, *LaMATE1* und *-2*. Wir haben untersucht, ob diese MATEs, wenn sie heterolog in Hefe exprimiert werden, entweder Citrat oder das Isoflavonoid Genistein transportieren können. Wir haben gesehen, dass LaMATE1 kein Citrat transportiert, aber LaMATE2 in isolierten Hefemikrosomen den Transport von Genistein katalysiert.

(iii) Um potentielle Kandidaten für den Flavonoidtransport in Arabidopsis zu identifizieren, haben wir uns auf publizierte Arbeiten bezogen und *in silico* Analysen durchgeführt. Wir haben drei Kandidaten für eine vertiefte Analyse ausgewählt, mit dem Ziel weitere im Flavonoidtransport involvierte MATEs zu identifizieren.

AtDTX35 (At4g25640) ist homolog zu einem Tomaten MATE Protein, beschrieben worden, das in einer Tomatenmutante hochreguliert ist, die den Transkriptionfaktor ANT1, der für die Anthocyanbiosynthese verantwortlich ist, überexprimiert (Mathews *et al*, 2003). Um herauszufinden, ob DTX35 an der Akkumulation von Anthocyanen beteiligt ist, haben wir i) DTX35 unter der Kontrolle des Banylus Promoters in der *tt12* Mutante exprimiert und ii) GUS Analysen durchgeführt, um *in silico* Daten zu validieren. Wir haben gesehen, dass DTX35 den Samenphänotyp von *tt12* nicht komplementieren kann und der Promotor in jungen Pflanzen überall aktiv ist.

AtDTX31 (At1g12950) wird in Wurzeln von Arabidopsis durch Salzstress induziert und zeigt eine Ähnlichkeit zu Proteinen, die in reifenden Tomaten stark exprimiert werden (Maathuis, 2006). Um zu bestätigen, dass AtDTX31 induziert wird, haben wir i) sqPCR Analysen mit Blatt- und Wurzelproben durchgeführt und ii) die Promotoraktivität in transgenen Arabidopsis Planzen analysiert, die ein *DTX31*-Promotor-Glucuronidase Konstrukt exprimieren. Wir können zeigen, dass DTX31 ein vakuolärer MATE ist, der unter Salzstress stark in Arabidopsiswurzeln exprimiert wird. Zusätzlich zeigen erste Experimente, dass in der entsprechenden Mutante die Wurzelexkretion verändert ist.

AtDTX28 (At5g44050) wurde als Gen beschrieben, das durch UV-B induzierbar ist, einem Stress, der bekanntermassen Gene induziert, die an der Favonolbiosynthese beteiligt sind, wie z.B. den Transkriptionsfaktor MYB12. Wir haben untersucht ob die Expression von *DTX28* in Arabidopsispflanzen verändert ist, die i) MYB12 überexprimieren, ii) die kein funktionelles MYB12 mehr besitzen und iii) bei denen drei MYBs, MYB11, MYB12 und MYB111, fehlen. Weitere Analysen beinhalteten HPLC Analysen um zu sehen, ob die Akkumulation von Flavonoiden in der *dtx28* Mutante verändert waren. Unsere Resultate zeigen, dass DTX28 kein verändertes Expressionsmuster in den verschiedenen Mutanten zeigt und dass das Flavonoidmuster nicht von demjenigen der Wildtyp Pflanzen abweicht.

CONTENTS

CHAPTER I: GENERAL INTRODUCTION

1.1. INTRODUCTION

Secondary metabolites play important roles in plant physiological processes. These roles include growth and development, reproduction and environmental adaptation. The term "secondary" was first proposed by Kossel in 1891 and suggested secondary metabolites to be present only in special, differentiated cells. Furthermore, their absence was proposed not to result in an abrupt cell death but in an impairment of survivability or no change at all. This is contrary to primary metabolites which are ubiquitous and essential for survival.

Plants produce a great variety of secondary products which are classified into three major groups: alkaloids, terpenoids and phenolic compounds (Hadacek, 2002) and some minor groups including glucosinolates, betalaines and cyanogenic glucosides. These groups are classified on the basis of their structure and biosynthetic pathways (Edreva *et al*, 2008). Among secondary metabolites small, simple molecules such as simple phenolics but also complex structures contributed by two different classes of secondary metabolites as in the case of vinblastine and vincristine can be found (Ishikawa *et al*, 1997; Ashutosh *et al*, 2006). Secondary metabolites have many diverse functions including major structural components, antimicrobial compounds (phenolic acids, flavonoids) or antioxidants (terpenoids, flavonoids; Beckman, 2000; Dixon *et al*, 2002; Velikova *et al*, 2005; Leopoldini *et al*, 2006; Vasconsuelo and Boland, 2007; Velikova *et al*, 2007).

The flavonoids belong to the group of phenolic compounds and are one of the largest classes of secondary metabolites constituting more than 9000 widely distributed water-soluble pigments. They are products of phenylpropanoid metabolism (Fig. 1.1). They are derived from two products: one of the general phenylpropanoid pathway (4-coumaroyl-CoA) and malonyl-coenzyme A, in a committed reaction catalyzed by chalcone synthase (CHS). Flavonoid biosynthesis is placed in the cytoplasm, mediated by enzymes which are associated with endoplasmic reticulum membranes where the large enzymatic complex is formed (Hrazdina *et al*, 1987). There are six major subgroups of flavonoids: colorless chalcones, flavones, flavonols, flavandiols, anthocyanins, proanthocyanidins and aurones. Additionally, three special groups are defined by their occurrence in specific plants: isoflavonoids occurring in legumes and a small group of non-legume plants; 3-deoxyanthocyanins occurring in *Sorghum bicolor, Zea mays* and *Sinningia cardinalis* and stilbenes occurring in *Vitis vinifera, Arachis hypogaea* and *Pinus silvestris* (Winkel-Shirley, 2001).

A number of physiological and biochemical functions have been assigned to the flavonoids which include pigmentation in flowers, leaves, fruits and seeds (Forkmann, 1993), key roles in male fertility in some species (Burbulis *et al*, 1996), signaling between plants and microbes (Shaw *et al*, 2006), UV protection (Kootstra, 1994) and in defence as potent antioxidants (Rice-Evans *et al*, 1997). They also play a role in free radical scavenging (Gao *et al*, 1999) and metal chelating (Leopoldini *et al*, 2006). Anthocyanins and flavonol pigments participate in the recruitment of pollinators and seed dispersion (Harborne and Williams, 2000).

The flavonoids have become one of the foci of research into phytocompounds with beneficial effects on human health. Arguably, one of the most impactive (and controversial) proposals in recent years came with the "French paradox" (the low cardiovascular mortality rate observed in populations connected to red wine consumption and high saturated fatty acid intake). It was suggested that the high abundance of flavonoids in red wine was correlated to the reduction in the risk of contracting coronary heart disease and that regular intake of flavonoids might reduce the risk of death from that disease in elderly men (Hertog *et al*, 1995).

Additionally, quercetin inhibits activities of cyclooxygenase and lipoxygenase which are responsible for inflammatory response, thus diminishing the formation of inflammatory metabolites (Robak and Gryglewski, 1996; Kim *et al*, 1998). It has been demonstrated that flavonoids such as apigenin or luteolin can inhibit cell proliferation thus it has been confirmed that as antioxidants they can inhibit carcinogenesis (Fang *et al*, 2007; Yoo *et al*, 2009). Flavonoids have been also shown to have antiviral effects. Kaul *et al* (1985) reported that herpes simplex virus, respiratory syncytial virus, parainfluenza virus and adenovirus are affected by flavonoids. Since the 1980s studies of the antiviral activity have been mainly focused on HIV. It has been shown that *in vitro* quercetin inhibits HIV-1 infection in peripheral blood mononuclear cells (Wang *et al*, 1998; Nair *et al*, 2009).

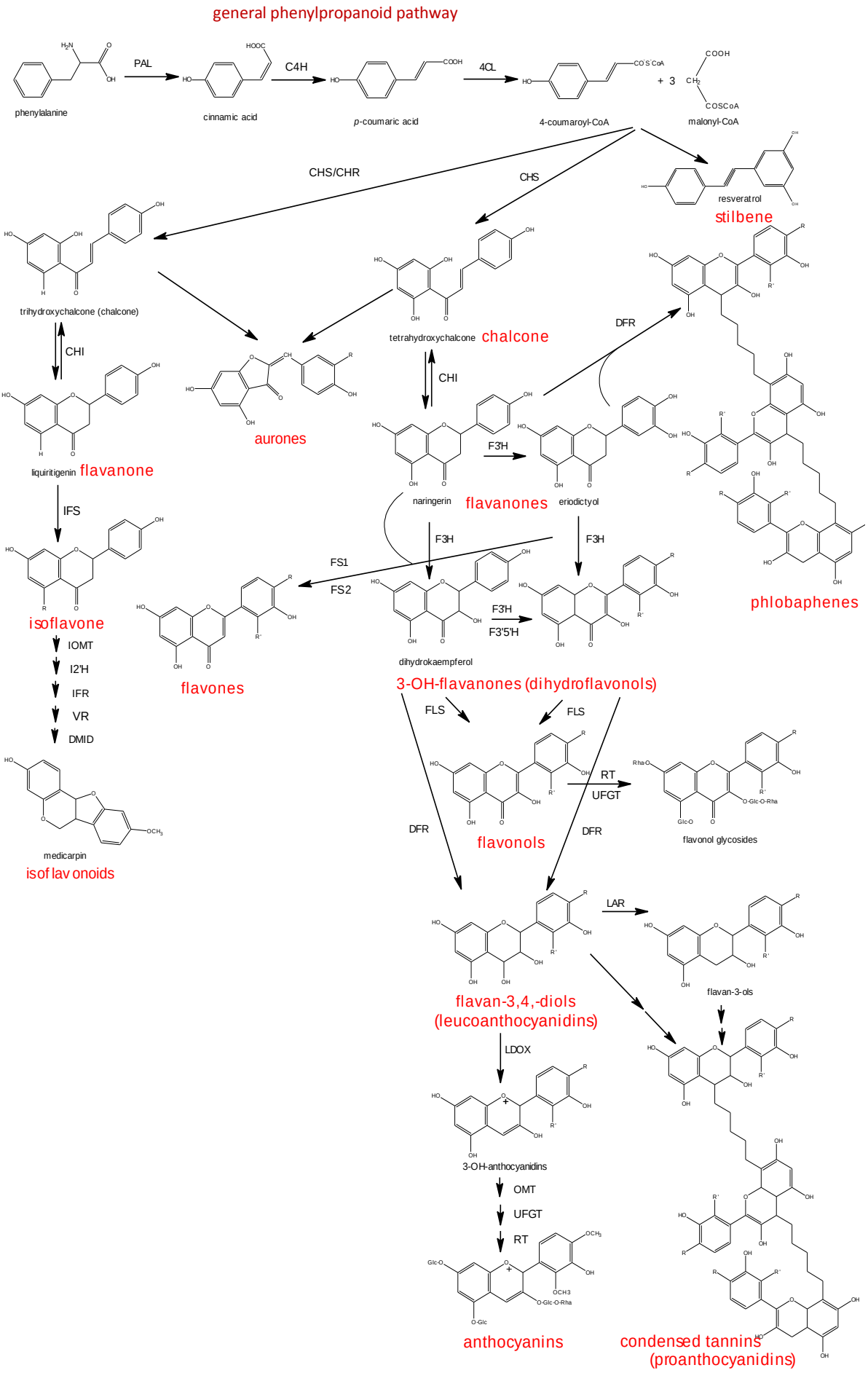

general phenylpropanoid pathway
PAL
C4H
4CL
+ 3
phenylalanine
cinnamic acid
p-coumaric acid
4-coumaroyl-CoA
malonyl-CoA
CHS/CHR
CHS
resveratrol
stilbene
trihydroxychalcone (chalcone)
tetrahydroxychalcone
chalcone
CHI
DFR
aurones
liquiritigenin
flavanone
F3'H
naringerin
flavanones
eriodictyol
phlobaphenes
IFS
FS1
FS2
F3H
F3'H
F3'5'H
isoflavone
flavones
dihydrokaempferol
3-OH-flavanones (dihydroflavonols)
IOMT
I2'H
IFR
VR
DMID
medicarpin
isoflavonoids
FLS
RT
UFGT
flavonols
flavonol glycosides
LAR
flavan-3-ols
flavan-3,4,-diols
(leucoanthocyanidins)
LDOX
3-OH-anthocyanidins
OMT
UFGT
RT
anthocyanins
condensed tannins
(proanthocyanidins)

Figure 1.1. Scheme of phenylpropanoid metabolism showing nine major groups of flavonoids: chalcones, aurones, isoflavonoids, flavones, flavonols flavandiols, anthocyanins, condensed tannins and phlobaphenes. Shortened enzyme names: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy,4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid3' hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H), isoflavone *O*-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LAR), *O*-methyltransferase (OMT), phenylalanine ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), vestitone reductase (VR). Scheme adapted from Winkel-Shirley (2001).

1.1.2. FLAVONOID BIOSYNTHESIS

The phenylpropanoid pathway is required to generate a wide variety of other secondary metabolites, such as lignins (cell wall component), stilbenes (anti- pathogenic, herbivory compound), phenolic acids (anti-pathogenic, antioxidant), lignans (estrogen-like and antioxidant) and flavonoids for flavonoid biosynthesis. The key substrates are (i) phenylalanine produced via the shikimate pathway and (ii) malonyl-CoA obtained by the carboxylation of acetyl-CoA catalyzed by the acetyl-CoA carboxylase. End products of flavonoid biosynthesis are transported to diverse subcellular or extracellular spaces, with those involved in pigmentation usually being moved into the vacuole (Andersen and Markham, 2006).

The first products of the flavonoid pathway, the chalcones, are developed from hydroxycinnamic acid-CoA esters, usually 4-coumaroyl-CoA, in three reactions involving malonyl-CoA. In the first one, phenylalanine is converted in a reaction catalysed by phenylalanine ammonia lyase (PAL) into cinnamic acid. In the second reaction this product undergoes reaction catalysed by cinnamate-4-hydroxylase resulting in formation of *p*-coumaric acid. The third reaction modifies p-coumaric acid to p-coumaroyl-CoA under the control of 4-coumarate:coenzyme A ligase (Winkel-Shirley, 2001).

Most enzymes operate in complexes located in the cytosol. The flavonoid production starts with the formation of the C15 backbone by chalcone synthase (CHS). Chalcones are then directly and indirectly modified to a range of other flavonoids (Davies and Schwinn, 2006) (Fig. 1.1).

1.1.3. MYB TRANSCRIPTION FACTORS REGULATE FLAVONOID BIOSYNTHESIS

The flavonoid biosynthetic genes are mainly coordinated at the level of transcription in response to biotic and abiotic stress (Czemmel *et al*, 2009). Based on their DNA-binding domains, transcription factors (TFs) can be categorized into various families or superfamilies (for review, Pabo and Sauer, 1992). The MYB TFs were categorized into three main groups: R2R3-MYB, R1R2R3-MYB and a heterogeneous group called the MYB-related proteins, which usually contain a single MYB repeat (Rosinski and Atchley, 1998; Jin and Martin, 1999; Stracke *et al*, 2001). All R2R3-MYB proteins share two highly conserved imperfect repeats at the N termini, whereas C termini are highly diverse (Stracke *et al*, 2001). Transcriptional regulators belong to the protein families containing R2R3-MYB domains, basic helix-loop-helix (bHLH) domains and WD repeats (Marles *et al*, 2003). In different plants, the combination and interaction of those TFs control the set of expressed genes (Baudry *et al*, 2004). There are 126 R2R3-MYB TFs in the Arabidopsis genome which are involved in various processes and thus the MYB TF family is the largest of the Arabidopsis TFs (Riechmann *et al*, 2000).

MYB-related TFs are confirmed to control diverse branches of flavonoid metabolism in plants and are thought to hold broader functions in the regulation of phenylpropanoid pathways in general (Tamagnone *et al*, 1998). Most R2R3-MYB TFs controlling flavonoid metabolism rely on cofactors, including WDR proteins and a small group of bHLH proteins which interact with a signature motif in the R3 repeat of the N-terminal R2R3 domain of MYB TFs (Grotewold *et al*, 1994).

Recently, the first R2R3-MYB genes: MYB11 (At3g62610), MYB12 (At2g47460), MYB111 (At5g49330) controlling flavonol synthesis have been reported in Arabidopsis (Mehrtens *et al*, 2005; Stracke *et al*, 2007). They are cofactor (bHLH) independent and regulate flavonol accumulation in various organs of developing seedlings. MYB12 primarily controls flavonol biosynthesis in roots, while MYB111 influences flavonol production in cotyledons. The triple mutant *myb11-12-111* does not accumulate flavonol glycosides but does accumulate anthocyanins, confirming the role of these three TFs only in flavonol accumulation (Stracke *et al*, 2007). All described MYB factors (MYB11, MYB12 and MYB111) are targeting CHS, CHI, FLS and FLS1 (Mehrtens *et al*, 2005; Stracke *et al*, 2007).

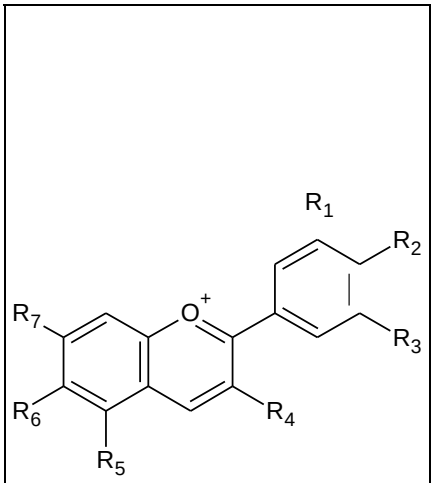
Another transcription factor PAP1/AtMYB75/At1g56650 was identified from an activation-tagging population of Arabidopsis. Constitutive overexpression of the cDNA in Arabidopsis caused an intensive purple pigmentation in most of the vegetative organs of transgenic lines. This was reported to occur as a consequence of a strong activation of the phenylpropanoid pathway resulting in significant hyperaccumulation of lignin, hydroxycinnamic acid esters and anthocyanins (Borevitz *et al*, 2000).

1.1.4. ANTHOCYANINS

Anthocyanins were most probably the first flavonoids to be studied because of their visibility and high concentrations. They constitute a major flavonoid group which, with some exceptions, is responsible for the color of most flowers, leaves, fruits and seeds of angiosperms and range from pink through red to dark blue (Andersen and Markham, 2006).

Structurally, the anthocyanins are composed of an aglycone (anthocyanidin), sugar(s) and in some cases also acyl group(s). Most occur as variations of only six anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Chopra *et al*, 2006) (Table 1.1.).

Table 1.1. Selected anthocyanidins and their substitutions (Kong *et al*, 2003)

Anthocyanidin	**Basic structure**	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**	**R_7**
Aurantinidin		−H	−OH	−H	−OH	−OH	−OH	−OH
Cyanidin		−OH	−OH	−H	−OH	−OH	−H	−OH
Delphinidin		−OH	−OH	−OH	−OH	−OH	−H	−OH
Europinidin		$-OCH_3$	−OH	−OH	−OH	$-OCH_3$	−H	−OH
Luteolinidin		−OH	−OH	−H	−H	−OH	−H	−OH
Pelargonidin		−H	−OH	−H	−OH	−OH	−H	−OH
Malvidin		$-OCH_3$	−OH	$-OCH_3$	−OH	−OH	−H	−OH
Peonidin		$-OCH_3$	−OH	−H	−OH	−OH	−H	−OH

Petunidin		−OH	−OH	$-OCH_3$	−OH	−OH	−H	−OH
Rosinidin		$-OCH_3$	−OH	−H	−OH	−OH	−H	$-OCH_3$

Common 3-hydroxy forms of anthocyanins rarely exist as aglycones because of the less stable structure and are immediately glycosylated at the C-3 position. The first stable products are 3-*O*-glycosides with glucose being the most common substituent. UDP-3-O-glucosyltransferases (UFGT) have been confirmed for different anthocyanins in seedlings, flower petals and seed coats (Harborne, 1994) They are able to glycosylate more than one type of anthocyanin B-ring and can also glycosylate flavonols (with exception of dihydroflavonols) (Stafford, 1990) .

One of the well-known functions of anthocyanins is in the production of flower color attractive to plant pollinators. Many factors including concentration and form of the anthocyanidin, metal complexes, pH, salts, etc. have been confirmed to have an influence on anthocyanins colors (Brouillard, 1994). Harborne and Williams (2000) reported that delphinidin is the most common anthocyanidin in blue flowers and that co-pigmentation with a flavone component is the most popular mechanism for shifting the violet colors of delphinidin glycosides toward blue.

Anthocyanins are typically synthesized in the cytoplasm, but like many other flavonoids, they may be transported across the tonoplast into the vacuolar space as shown in maize (Marrs *et al*, 1995), grapevine (Gomez *et al*, 2009) and *Medicago truncatula* (Zhao and Dixon, 2009). They have also been found in cytoplasmic vesicles called anthocyanoplasts (APIs) which are completely omitted in the vacuole and might be responsible for transporting anthocyanic vacuolar inclusions (AVIs) into that cell compartment (Braidot *et al*, 2008). The presence of AVIs in cells was shown to have a significant influence on flower color by enhancing its intensity (Markham *et al*, 2000). Zhang and his co-workers (Zhang *et al*, 2006; Zhang *et al*, 2007) reported that AVIs might be membrane-delimited and contain a high amount of mix acylated anthocyanins, tannins and organic compounds.

Anthocyanin accumulation may be induced by sugars in many plants (Teng *et al*, 2005). It has been shown that Arabidopsis mutants with altered reactions to sugar also demonstrate altered sugar-induced anthocyanin accumulation (Mita *et al*, 1997, 1997; Baier *et al*, 2004). Teng and his co-workers (2005) reported that sucrose-dependent signaling pathway causes anthocyanin accumulation in a process that depends on a MYB75/PAP1 TF.

1.1.5. FLAVONOLS

Flavonols represent one of the most abundant classes of flavonoids and accumulate in a large variety of conjugate structures (Jones *et al*, 2003). Most plants synthesize derivatives of the three major flavonols: quercetin, kaempferol and myricetin, which vary by only a single hydroxyl group on the B ring and yet play important roles in different biological processes (Owens *et al*, 2008).

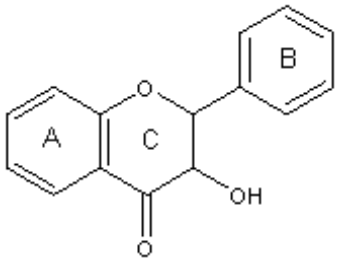


Figure 1.2. General flavonol structure (Aherne and O'Brien, 2002)

The flavonoids consist of three phenolic rings referred to as the A, B and C rings (Kühnau, 1976). Ring C may be a heterocyclic pyran (flavanols, anthocyanidins) or pyrone (flavones, flavanones). Flavanols, flavanones, flavonols and flavones are often called 4-oxo-flavonoids because each class of these compounds carries a carbonyl group on C-4 of ring C (Fig. 1.2).

It has been demonstrated that flavonols protect plants against UV light. It has been shown that UV-B elevates concentration of quercetin glycosides in grapevine (Price *et al*, 1995; Downey *et al*, 2007), petunia (Ryan *et al*, 2002), mustard (Beggs *et al*, 1987) and soybean (Middleton and Teramura, 1993) whereas a flavonol synthase Arabidopsis mutant is unable to produce flavonols in response to UV radiation (Wisman *et al*, 1998).

Flavonols are considered to play a role in the regulation of auxin transport and combined with kaempferol exhibit different spatial and temporal distribution patterns in *Arabidopsis thaliana* roots which confirms the role of flavonols in that process (Peer *et al*, 2001; Peer *et al*, 2004; Santelia *et al*, 2008).

Flavonols were also identified as effective compounds required to the creation of functional pollen tubes (Mo *et al*, 1992), pollen germination in tobacco and pollen tube growth *in vitro* (Ylstra *et al*, 1992).

1.1.6. PROANTHOCYANIDINS

Proanthocyanidins (PA), also called condensed tannins, are oligomeric and polymeric final products of the flavonoid biosynthesis that appear extensively in fruits, bark, leaves and seeds of plants (Dixon *et al*, 2005) (Fig 1.3). In *Arabidopsis* testa, PAs are called procyanidins and consist of the flavan-3-ol, 2,3-cis-(-)-epicatechin (Fig.1.3) (Abrahams *et al*, 2003).

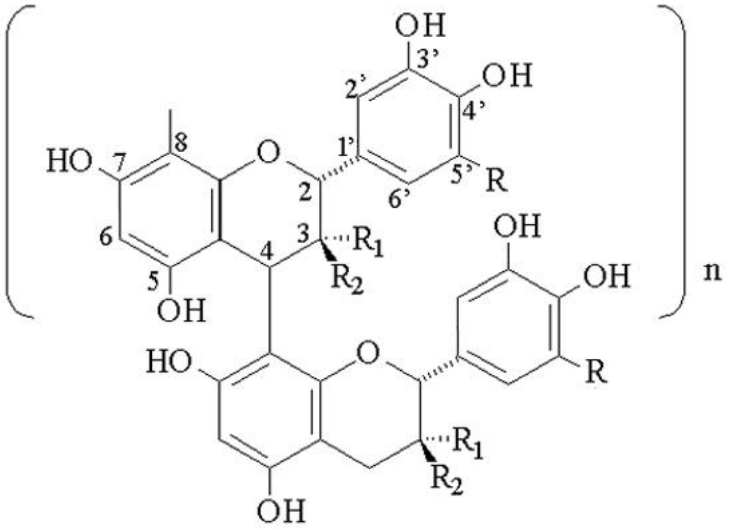


Figure 1.3. Chemical structure of proanthocyanidins. Where R = H, it is a procyanidin: catechin (R_1 = H and R_2 = OH) and epicatechin (R_1 = OH and R_2 = H); Where R = OH, it is a prodelphinidin: gallocatechin (R_1 = H and R_2 = OH) and epigallocatechin (R_1 = OH and R_2 = H). Image was taken from Garbacki *et al* 2004.

PAs play a role in defense against plant diseases and in seed dormancy (Debeaujon *et al*, 2000; Peters and Constabel, 2002). Colorless condensed tannins are synthesized in vesicles made from endoplasmic reticulum which further merge into the central vacuole (Stafford, 1988). They are oxidized into brown complexes in dead cells. PAs function also as potential antioxidants with benefits for human health, including protection against cardiovascular disease and against free radical-mediated injury (Bagchi *et al*, 2000; Middleton *et al*, 2000; Cos *et al*, 2004).

The first committed step in PA biosynthesis is catalyzed by the leucoanthocyanidin reductase (LAR) from 3, 4-*cis*-leucocyanidin (Tanner *et al*, 2003). PAs accumulate within the vacuole, therefore the initiating and extension units of the polymer must be transfered to that cell compartment. Even though the vacuole is the main storage place for PAs, it has been

reported that cells contain also PA-containing provacuoles which are originating from the rough endoplasmic reticulum (Parham and Kaustinen, 1977). It appears that small provacuoles may undergo fusion with the large central vacuole.

In *Arabidopsis*, the biosynthetic pathway leading to PA accumulation has been comprehensively described using reverse genetic approaches, specifically exploiting the *transparent testa* (*tt*), *tannin-deficient seed* (*tds*) and *banylus* (*ban*) mutants that all fail to accumulate PAs in their seed coat (Shirley *et al*, 1995; Abrahams *et al*, 2003). In the context of this dissertation these mutants will be elaborated upon further, with a specific emphasis on the *transparent testa* mutants.

1.2. TRANSPARENT TESTA MUTANTS

Flavonoid-deficient mutants offer a unique functional tool to analyze the roles of flavonoids in plant growth and development because null mutations in *TT* genes result in the alteration of pigments (PAs) in the seed coat (Shirley *et al*, 1995; Abrahams *et al*, 2002; Abrahams *et al*, 2003). Transparent testa (*tt1-tt19*), *transparent glabra* (*ttg, ttg1, ttg2*), *tannin deficient seeds* (*tds*) and *banylus* (*ban*) mutants all belong to the group of seed coat mutants which display this pigment alteration (Table 1.2) (Winkel-Shirley, 2001; Shikazono *et al*, 2003).

Table 1.2. Characteristics of *Arabidopsis thaliana testa* mutants.

Genotype	Seed color	Encoding	Subcellular localization	Tissue	Function	Phenotype	Reference
tt1	yellow	WIP zinc finger protein	nucleus	developing ovules, young seeds	affects the pigmentation of seed coat	when over-expressed it is causing aberrant growth and organ morphology	Sagasser *et al*, 2002
tt2	yellow	R2R3 MYB domain	mainly nucleus	seed during early embryoge-nesis	inducing *BAN* expression in young seedlings and roots	pigmentation reduced in seeds but intact in vegetative tissue	Nesi *et al*, 2001
tt3	yellow	dihydro-	cyto-	n.d.	n.d.	n.d.	Shirley *et al*,

		flavonol 4-reductase (DFR)	plasm				1992
tt4	yellow	chalcone synthase (CHS)	n.d.	n.d.	n.d.	shows a delay in the gravity response and cannot produce flavonoids; root looping	Koornneef, 1990 Buer and Muday, 2004
tt5	yellow	chalcone isomerase (CHI)	n.d.	n.d.	n.d.	increased density of lateral roots	Shirley *et al*, 1992
tt6	pale brown	flavonone 3-hydroxylase (F3H)	n.d.	n.d.	n.d.	increased density of lateral roots; longer hypocotyls than WT	Wisman et al, 1998; Pelletier and Shirley, 1996
tt7	pale brown	flavonoid 3'-hydroxylase (F3'H)	n.d.	mainly in siliques	n.d.	n.d.	Schoenbohm *et al*, 2000
tt8	yellow	MYC/bHLH domain protein	n.d.	n.d.	modulates the expression of DFR, controls marginal trichome development	long petiole	Nesi *et al*, 2000
tt9	grayish brown	n.d.	n.d.	n.d.	affects seed pigmentation	pigmentation reduced in seeds but intact in vegetative tissue	Shirley *et al*, 1995
tt10	pale brown	laccase-like protein	n.d.	mainly developing siliques and seeds	involved in oxidative polymerization of flavonoids in the seed coat	root looping	Pourcel *et al*, 2005
tt11	pale brown	n.d.	n.d.	n.d.	affect anthocyanin accumulation in vegetatitve plant parts	n.d.	Debeaujon *et al*, 2000
tt12	pale brown	MATE protein	vacuole	seed	vacuolar accumulation of PAs precursors in seeds	n.d.	Debeaujon *et al*, 2001
tt13	pale brown	n.d.	n.d.	n.d.	affects seed pigmentation	n.d.	Debeaujon *et al*, 2000
tt14	pale brown	n.d.	n.d.	n.d.	affects anthocyanin accumulation in vegetative parts	n.d.	Debeaujon *et al*, 2000
tt15	pale greenish-brown	none	n.d.	n.d.	n.d.	pigmentation reduced in seeds but intact in vegetative tissue	Focks *et al*, 1999

tt16	yellow	ARABIDO-PSIS BSISTER MADS domain (ABS) TF		expressed mainly in the buds, flowers and immature seeds	regulates organ and cell development for PAs deposition, controls cell shape	n.d.	Nesi *et al*, 2002
tt17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
tt18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
tt19	n.d.	GST family	n.d.	seed	participate in the uptake of PA precursors	n.d.	Walker *et al*, 1999
ttg1	yellow	WD40 repeat domain protein	n.d.		interacts with MYB and MYC TFs, controls trichome initiation and PAs production	n.d.	Ishida *et al*, 2007
ttg2	brown	WRKY TF	n.d.	young leaves, trichomes, seed coats and root hairless cells	modulates trichome differentiation, mucilage production and PAs biosynthesis	n.d.	Johnson *et al*, 2002
banylus	grayish-green	anthocyanin reductase (ANR)	cyto-plasm	seed coat	n.d.	n.d.	Devic *et al*, 1999
aha10	n.d.	P-type H^+-ATPase	central vacuole	developing seeds	n.d.	n.d.	Baxter *et al*, 2005

n.d. not determined

Recently using a reverse genetic approach, Arabidopsis TT12 was demonstrated to encode for an H^+/ flavonoid antiporter (Marinova *et al*, 2007). In that study TT12 was demonstrated to localize to the tonoplast and when heterologously expressed in yeast, vesicles were able to transport the cyanidin 3-glucoside (C3G). Coupled to the initial characterization of the T-DNA insertion mutant (*tt12*) which displays no accumulation of PAs in the seed coat (Debeaujon *et al*, 2001), a definitive physiological function could be assigned to TT12. Follow up work on the amino acid residues responsible for the transport activity of TT12 is described in Chapter III of this dissertation.

1.3. MEMBRANE TRANSPORTERS AND SECONDARY METABOLITES

To achieve their function/s, secondary metabolites generally accumulate to high concentrations in different tissues and/or cell types. Storage in suitable compartments suggests that this process is highly regulated, since some of the secondary metabolites (e.g. berberine) are toxic to the plants themselves (Yazaki, 2005). Secondary metabolites are transported in various ways, either between tissues or within a cell. Intercellular and intracellular transport is mediated mostly by specific transporters (Yazaki *et al*, 2008).

Vacuoles play a central role in the storage of secondary metabolites such as alkaloids and flavonoids in plant cells. Vacuolar membranes (tonoplasts) contain a large number of transporters, channels and pumps (Martinoia *et al*, 2007). Since modified flavonoids are hydrophilic, it must be assumed that their *intra*- and *inter*- cellular transport depends heavily on membrane bound transporters.

1.3.1. VARIOUS TYPES OF MEMBRANE TRANSPORTERS ARE IMPLICATED IN SECONDARY METABOLITE TRANSPORT

Alkaloids (nitrogen-containing organic compounds, mostly aromatic) are often sequestrated in the vacuoles to neutralize their toxic effect (Hashimoto and Yamada, 2003). The characterization of the cellular uptake of berberines (a yellow isoquinoline alkaloid widely used as an antibacterial and antimalarial drug), into cell cultures of *Coptis japonica* implied the involvement of an ABC transporter (Sakai *et al*, 2002).

Terpenoids are the largest group of secondary metabolites formed from prenyl diphosphates of different chain length provided by the elongation of five-carbon isoprene units (Croteau *et al*, 2000). A pleiotropic drug resistance (PDR/ABCG)-type ABC transporter from *Nicotiana plumbaginifolia* is involved in the secretion of diterpene and sclareol onto the leaf surface (Jasinski *et al*, 2001).

Flavonoid transport has been suggested to occur via two distinct mechanisms: membrane vesicle-mediated and membrane transporter-mediated. However, vesicle-mediated transport also requires a transporter-mediated step which facilitates the loading of vesicles with the flavonoids or their precursors (Zhao and Dixon, 2010).

1.3.2. MEMBRANE VESICLE-MEDIATED TRANSPORT OF FLAVONOIDS

Membrane vesicle-mediated transport evolved from microscopy observations. At first anthocyanoplasts, cytoplasmic anthocyanin bodies surrounded by a membrane and originating from many vesicle-like structures fused together were considered to be transfer vesicles or sites of anthocyanin biosynthesis (Grotewold, 2004; Markham *et al*, 2000; Braidot *et al*, 2008). They are covered by a membrane and derived from a fusion of number of smaller vesicle-like structures. Similar structures, anthocyanic vacuolar inclusions (AVIs) were observed in the vacuole and also showed some movement (Irani and Grotewold, 2005). They seemed to be involved in the storage function rather than anthocyanin transport and even though they are associated with membranous substances and proteins, they are not enclosed by a membrane (Fig. 1.4). Conversely cytoplasmic vesicle-like structures enclosing anthocyanins have been discovered to associate with AVIs after they merge with the vacuole (Zhang *et al*, 2006). These structures might be transferred into the central vacuole through fusion of prevacuolar compartments (PVCs) and then the central vacuole (Fig. 1.4). Additionally, anthocyanin-containing vesicle-like structures can co-localize with protein storage vacuoles (PSVs) and transfer anthocyanins in *trans*-Golgi network (TGN)-independent from ER-to-PVC vesicle trafficking pathway (Poustka *et al*, 2007) (Fig. 1.4).

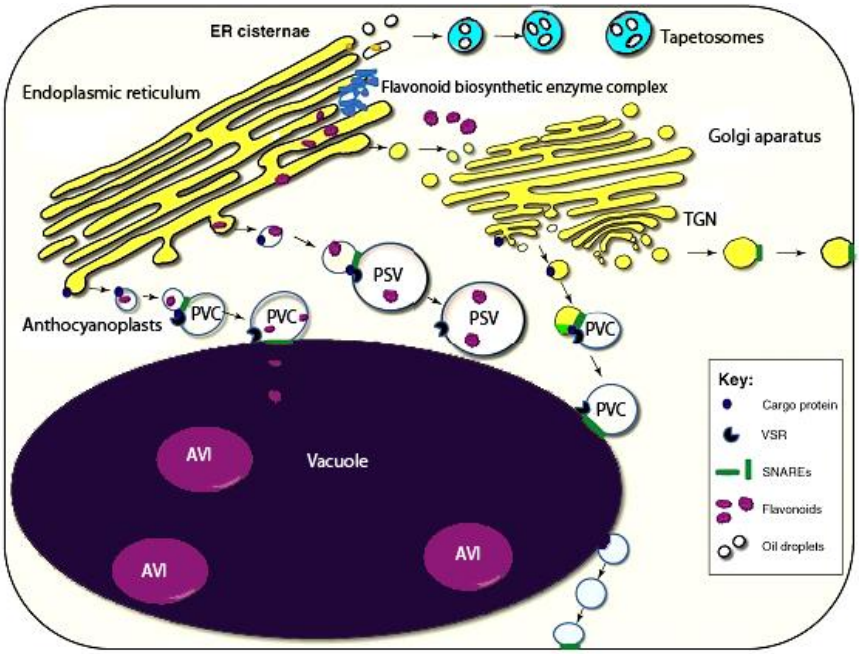


Figure 1.4. Vesicle-mediated transport of flavonoids. Adapted from Zhao and Dixon, 2009.

The tapetosomes of *Arabidopsis* anthers are known as specialized structures containing ER-derived flavonoids which are delivered to the pollen surface supporting the idea that flavonoids can be relocated from the ER via cytoplasmic vesicles (Hsieh and Huang, 2007) (Fig. 1.4).

Recently, it has been reported that *Arabidopsis transparent testa* mutants also show morphological defects in the central vacuole of the seed coat endothelium cells. No central vacuole present and PA storage limited to the prevacuole-like small vesicles was observed in *tds4* mutant (Abrahams *et al*, 2003). Comparable alterations have been observed in *tt19* (glutathione *S*-transferase, GST), *aha10* and *tt12* mutants (Kitamura *et al*, 2004; Baxter *et al*, 2005). As a result, it has been suggested that PA accumulation and vacuole biogenesis might be closely related or PA precursors are transferred through ER-originated vesicles to the vacuole. However, *ph5* mutant of the tonoplast-localized H^+-ATPase from *Petunia hybrida* did not show abnormal vacuoles, while *PH5* is an ortholog of *AHA10* and is implicated in the vacuolar PA accumulation in the seed coat (Verweij *et al*, 2008).

Thus far many researchers have shown involvement of GSTs in anthocyanin accumulation (Marrs *et al*, 1995; Alfenito *et al*, 1998, Peel *et al*, 2009). Biochemical and genetic studies demonstrated that GST protein is essential for anthocyanin sequestration (Larsen *et al*, 2003). GST can bind to anthocyanin or flavonol to form complexes which suggest that GSTs guard flavonoids from oxidation and/or guide them to the central vacuole (Mueller *et al*, 2000).

1.3.2. MEMBRANE-TRANSPORT MEDIATED TRANSPORT OF FLAVONOIDS

1.3.3.1. ATP binding cassette (ABC) transporters

The largest protein family known, ATP-binding cassette transporters are multi-domain membrane proteins that utilize energy from ATP hydrolysis to transfer substrates across cellular membranes (Higgins, 1992; Henikoff *et al*, 1997; Sánchez-Fernández *et al*, 2001, Yazaki, 2006). This protein superfamily has over 120 members in both *Arabidopsis thaliana* and rice (*Oryza sativa*) but only a few of them have been described. These characterised ABCs show diverse transport activities toward a variety of substrates including auxins, xenobiotics, metal ions, malate etc. (Rea, 2007).

1.3.3.1.1. ABC transporters and secondary metabolites

Most of the phenolic compounds are detected in glycosylated form. Glucosidation is essential for detoxification of endogenous secondary metabolites which usually are stored in the vacuoles. Multidrug resistance-associated protein (MRP or ABCC)-type ABC transporters are suggested to be involved in the vacuolar sequestration of glucosides (Bartholomew *et al*, 2002; Frangne *et al*, 2002). Few genes of the large group of MRP genes in *Arabidopsis* have been characterized in more detail. *AtMRP1*, *AtMRP2*, *ATMRP3, ATMRP4* and *ATMRP5* encode functional GS-conjugate pumps and transfer secondary metabolites into the vacuole (Lu *et al*, 1997; Lu *et al*, 1998; Tommasini *et al*, 1998). However, it should be mentioned that GS conjugates of anthocyanins as used in experiments with AtMRP2 have never been observed *in vivo*.

In petunia, the gene *An9* encodes a Phi-type GST involved in the vacuolar uptake of anthocyanins (Alfenito *et al*, 1998). It has been suggested that *AN9* is not the final gene in the anthocyanin pathway though and it is controlled by *AN1*, *AN2* and *AN11* and some additional gene (acting after *AN9*) conferring cell autonomy. Latest reports suggest that there are vacuolar transporters involved in this process which belong to the family of ABC or MATE transporters (Kitamura, 2006). Additionally, the step of the pathway controlled in petunia by the *An9* gene, is controlled by *Bz2* gene in maize. The Arabidopsis *TT19* locus also encodes a Phi-type GST and maintains the accumulation of anthocyanins and PAs. The overexpression of the petunia *An9* gene in the Arabidopsis *tt19* mutant complemented the anthocyanin accumulation phenotype but not PAs pigmentation in the seed coat (Kitamura *et al*, 2004). The involvement of these GSTs in the transport of anthocyanins to the vacuole is still unknown. GS-anthocyanin conjugates have never been detected in plant extracts and as a result, the roles of GST alternative to glutathionation of the anthocyanins are being investigated (Kitamura, 2006; Gerats and Strommer, 2009).

Since MRPs have a substrate preference to glutathione conjugates and their transport activity is stimulated in the presence of glutathione, it has been implicated that MRPs are connected to the vacuolar transport of anthocyanins (Marrs *et al*, 1995). Further proof of MRPs being involved in anthocyanin accumulation was given by Goodman and his co-workers (2004). They showed that ZmMRP3 is localized to the tonoplast and essential for the anthocyanin accumulation in maize. The loss of function mutant *mrp3* shows reduction in the anthocyanin level and in the accumulation of brown pigments in the cytoplasm. It is localized at the tonoplast which confirms the hypothesis that BZ2 from maize may transport C3G to

the vacuolar membrane to assist their uptake by MRP3 (Goodman *et al*, 2004), although there is no biochemical or functional evidence that ZmMRP3 acts a transporter.

1.3.3. MULTIDRUG AND TOXIC EXTRUSION (MATE) TRANSPORTERS

The MATE family includes a large group of secondary transporters that are widely represented in all living organisms from prokaryotes to eukaryotes (Brown *et al*, 1999; Moriyama *et al*, 2008). MATE transporters range in size of 400-700 amino acids with 9-12 transmembrane α-helices but no consensus sequences have been identified. They use the electrochemical proton or sodium gradient generated by proton pumps in plants and K^+/Na^+ ATPases in animals (Gaxiola *et al*, 2002). The detoxification of xenobiotics and transport of a wide range of metabolites such as cations, organic acids and secondary metabolites are the main function of the plant MATE transporters (Yazaki, 2005).

The first MATE protein – NorM, was identified in *Vibrio parahaemolyticus*. It has been reported as sodium – multidrug antiporter which conferred resistance to norfloxacin (widely used as an antimicrobial compound) (Morita *et al*, 1998; Morita *et al*, 2000).

Thus far putative MATE transporters sequences have been identified across the taxonomic kingdoms and some of their functions have been clarified (Omote *et al*, 2006). The human MATE family contains two homologous genes *MATE1* and *MATE2*. *MATE1* is highly expressed in the liver and kidneys and transports tetraethylammonium when expressed in HEK293 cells (Otsuka *et al*, 2005a). *MATE2* has been identified as a transporter of various organic cations (Masuda *et al*, 2006). Discovery of a rodent MATE1 transporter (Hiasa *et al*, 2006) together with human MATEs findings imply that mammalian MATE transporters represent the polyspecific organic cation exporters catalyzing the terminal transport of toxic cations into urine and bile (Klaassen and Aleksunes, 2010).

The X-ray structure of the MATE transporter NorM from *V. cholerae* revealed an outward-facing confirmation with two portals open to the outer leaflet of the membrane and a topology of the predicted 12 transmembrane α helices forming two bundles of six transmembrane helices shaping a large internal cavity. It has been reported that there is a monovalent cation-binding site, in close proximity to residues previously described as critical for transport. The two halves (bundles) on one side are connected by a cytoplasmic loop whereas the initial α helix of each half is united by a helical extension from the inner membrane leaflet side (He *et al*, 2010).

Arabidopsis contains 56 putative MATE transporter genes in its genome, although the functions have been only reported for some members (Table 1.3).

Table 1.3. Properties of plant MATE-type transporters. Modified from Yazaki *et al*, 2008.

Plant	Name	Driving force	Subcellular localization	Tissue	Substrate	Proposed physiological function	Reference
Arabidopsis	*ALF5**	n.d.	n.d.	Root (epidermis, cortex)	(TMA, PVP)	Protection of the root from toxic compounds	Diener *et al*, 2001
	DTX1	H^+	PM	Flower, leaf, stem, root	(Norfloxacin, EtBr, berberine, heavy metals)	Efflux of endogenous metabolites and xenobiotics	Li *et al*, 2002
	*EDS5**	n.d.	n.d.	n.d.	n.d.	Salicylic acid-dependent signaling cascade for disease resistance	Nawrath *et al*, 2002
	*FRD3**	n.d.	n.d.	Root (percycle, vascular cylinder)	Citrate	Efflux of citrate into the root vasculature for iron translocation	Rogers and Guerinot, 2002; Green and Rogers, 2004
	AtMATE (AtFRDL)	n.d.	n.d.	Root	Citrate	Al activated root citrate exudation into the rhizosphere	Liu *et al*, 2009

	TT12	H^+	Vac	Seed coat endothelium	Flavonoids	Vacuolar flavonoid/H^+ antiporter in the seed coat	Debeaujon *et al*, 2001; Marinova *et al*, 2007
	*FFT**	n.d.	n.d.	Young seedlings, senescent leaves, roots	n.d.	Required for correct reproductive development	Thompson *et al*, 2010
Tomato	*MTP77*	n.d.	n.d.	n.d.	n.d.	Vacuolar transporter of anthocyanins (similar to TT12)	Mathews *et al*, 2003
Lupin	*LaMATE*	n.d.	PM	Root	n.d.	n.d.	Uhde-Stone *et al*, 2005
Barley	*HvMATE*	n.d.	n.d.	n.d.	(Citrate)	Aluminum tolerance	Wang *et al*, 2007
	HvAACT1	n.d.	PM	Root, shoot	Citrate	Al-activated efflux carrier of citrate, alumnium tolerance	Furukawa *et al*, 2007
Sorghum	*SbMATE*	n.d.	PM	Root	(Citrate)	Al-activated efflux carrier of citrate, aluminum tolerance	Magalhaes *et al*, 2007
Grapevine	*anthoMATE*	H^+	Vac	Mature berries	Acylated anthocyanins	Vacuolar transporter of anthocyanins	Gomez *et al*, 2009
Tobacco	*Nt-JAT1**	H^+	Vac in leaves, PM in roots	Leaf, stem, root	Alkaloids	Nicotine transporter	Morita *et al*, 2009

Medicago truncatula	*MtMATE1*	H^+	Vac	Seed coat	Epicatechin 3'-*O*-glucoside	Vacuolar transporter of E3'G for PAs biosynthesis	Zhao and Dixon, 2009
Rice	*OsFRDL1*	n.d.	PM	Pericycle cells of the roots	Citrate	Iron translocation as a Fe-citrate complex	Yokosho *et al*, 2009

*ALF5 - aberrant lateral root formation; EDS5 – enhanced disease susceptibility; FRD3–ferric reductase defective; FFT – flower flavonoid transporter; JAT1 – jasmonate-inducible alkaloid transporter 1; FRDL1- FRD3 like 1
n.d. not determined, PM - plasma membrane, Vac - vacuole, TMA - tetramethylammonium, PVP - polyvinylpyrrolidone, EtBr - ethidium bromide

As shown in table 1.3, in Arabidopsis, *AtFRD3* and *AtMATE* encode citrate transporters which play roles in different biological processes. *AtFRD3* is involved in loading citrate into xylem tissues which facilitates iron transport to shoots (Rogers and Guerinot, 2002; Green and Rogers, 2004; Durrett *et al*, 2007), while *AtMATE* is responsible for protection of roots from Al toxicity by exudating citrate into rhizosphere (Liu *et al*, 2009).

Transparent testa 12 (TT12) has been shown to be involved in the vacuolar accumulation of PAs precursors in the seed. It transports simple glycosylated compounds (flavan-3-ols) *in vivo* (Marinova *et al*, 2007). Similarly, in *Medicao truncatula*, *MtMATE1* transports epicatechin 3'-O-glucoside which is a precursor for PAs biosynthesis and is able to complement the seed phenotype of the Arabidopsis *tt12* mutant (Zhao and Dixon, 2009).

Yeast vesicles expressing *AM1* and *AM3* from *Vitis vinifera* transported acylated anthocyanins in the presence of MgATP. The inhibition studies have shown that the AM1 and AM3 proteins function *in vitro* as vacuolar H^+-dependent acylated anthocyanin transporters. It has also been demonstrated that these transporters did not mediate the uptake of non-acylated anthocyanin glucosides, suggesting that the acyl conjugation was essential for the transport (Gomez *et al*, 2009).

Function and activity of MATE transporters depend on several types of H^+-ATPases. V-type H^+-ATPase or vacuolar pyrophosphatase (V-PPase) proton pumps, sustain the proton gradient across the tonoplast (Verweij *et al*, 2008). Maintenance of the proton gradient across the plasma membrane is provided by P-type H^+ -ATPases (Gaxiola *et al*, 2002). Mutations of some of these MATEs result in flower color change and seed phenotypes similar to Arabidopsis *tt* mutants. The *Arabidopsis* AHA10 mutant which is a knockout of the P-type

H^+ -ATPase results in reduced PA content and a *tt* seed phenotype (Baxter *et al*, 2005). The *PH5* gene from petunia encoding H^+ -ATPase appears to be regulating the pH of the vacuolar lumen and therefore when mutated it causes changed flower color and reduced anthocyanin/PA production (Verweij et al, 2008)

Import and export of natural substances across the membranes can be maintained by different mechanisms. For instance, the import of substances into the vacuole typically happens with conjugated forms, while export of the same substances does not seem to require conjugation. Flavonoids are often modified upon biosynthesis and undergo glycosylation, hydroxylation, acylation and methylation. These modifications are essential for changing their solubility or stability and reducing toxicity (Zhao and Dixon, 2009). Acylation of anthocyanins is a requirement for their uptake through the MATEs described so far though glycosylation is adequate to facilitate the vacuolar uptake of many flavonoids (Klein *et al*, 1996, Klein *et al*, 2000; Frangne *et al*, 2002; Bartholomew *et al*, 2002; Marinova *et al*, 2007a; Marinova *et al*, 2007b).

1.4. THE ARABIDOPSIS MATE TT12 ACTS AS A VACUOLAR FLAVONOID/H+ ANTIPORTER

The TT12 gene (AtDTX41, At3g59030) was identified in the forward screen of T-DNA insertion lines and is the first transporter-like membrane protein participating in the PA biosynthesis. The *tt12* mutant seeds lack the flavan-3-ol epicatechins and PAs in the vacuoles of the seed endothelium (Debeaujon *et al*, 2001). TT12 belongs to the multidrug and toxic extrusion (MATE) family (Brown *et al*, 1999) and consist of 12 transmembrane α-helices (Schwacke *et al*, 2003). It has been demonstrated that TT12 acts as a vacuolar flavonoid/H^+ antiporter. The expression of TT12 is consistent to the defined expression pattern of *BANYLUS (BAN)* promoter which has been noted in the seed coat cells actively producing PAs (Albert *et al*, 1997; Devic *et al*, 1999; Debeaujon *et al*, 2003). Stable and transient expression of full length TT12 cDNA demonstrated a clear localization of TT12 to the tonoplast (Fig. 1.5 A) (Marinova *et al*, 2007). ProTT12:GUS fusion revealed that TT12 is specifically expressed in the seed coat endothelium (Fig. 1.5 B). LC-MS analysis showed that no soluble flavan-3-ols and PAs were present in mature seeds and that only the insoluble fraction of PAs was present. Furthermore vesicle uptake experiments carried out with

transformed yeast expressing TT12 revealed that TT12 is able to mediate cyanidin 3-glucoside uptake in the presence of ATP (Fig. 1.5 C).

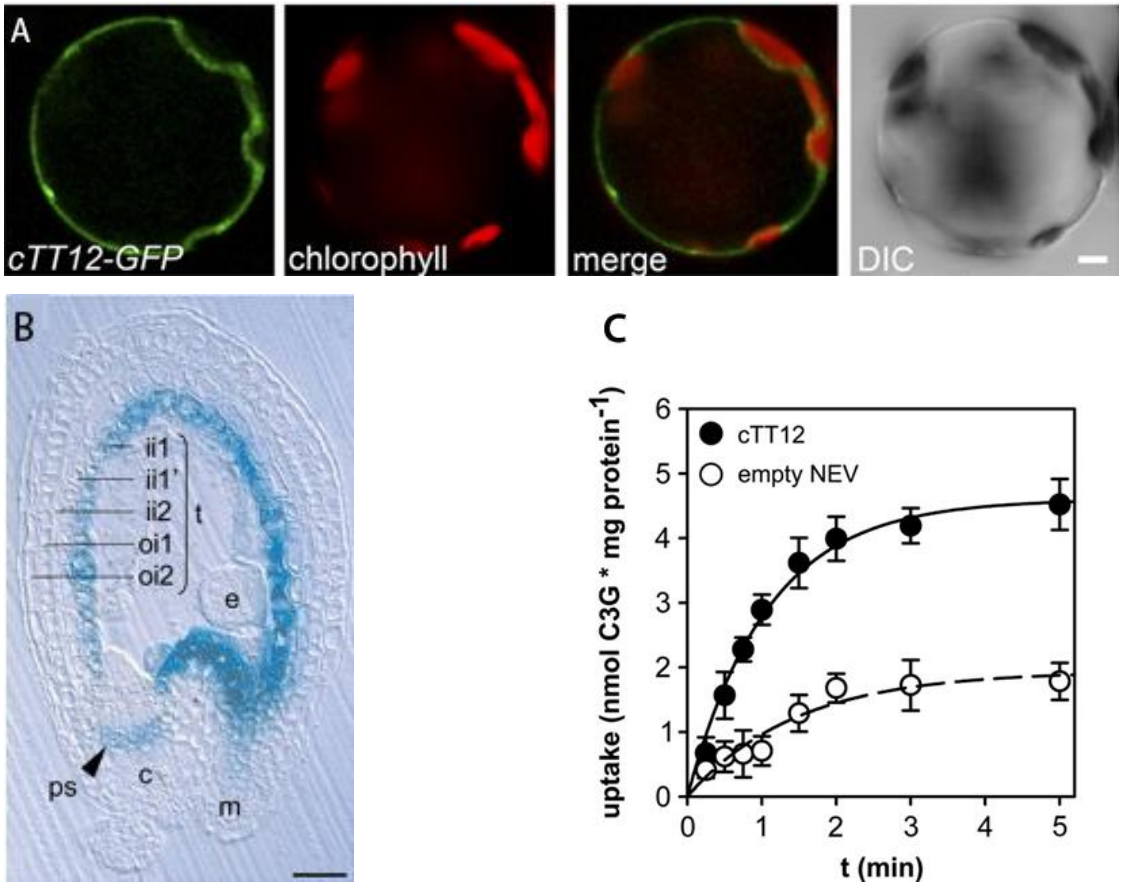


Figure 1.5. Characterization of TT12 gene (A) subcellular localization of the TT12 protein after transient expression; mesophyll protoplasts isolated after transient expression of cTT12-GFP, DIC differential interference contrast. Bars = 5µM; (B) activity of the TT12 promoter in wild-type developing seeds of Arabidopsis, 6 DAF. GUS activity was observed with Nomarski optics on section. The layer ii1 is also called endothelium; c, chalaza; e, embryo; ii, inner integument; m, micropyle; oi, outer integument; ps, pigment strand; t, testa. Bar = 33 µM. (C) TT12-mediated transport of C3G in yeast microsomal vesicles. Time-dependent uptake of C3G into vesicles isolated from yeasts transformed either with TT12 (closed symbols) or the empty vector (open symbols) in the presence of MgATP. Images were taken from Marinova *et al*, 2007.

Functional characterization where amino acids essential to the transport functions of MATE transporters were identified, has been reported for the human MATE1 and 2-K (Otsuka *et al*, 2005), NorM from *Vibrio parahaemolyticus* (Otsuka *et al*, 2005) and NorM from *Vibrio cholerae* (Singh *et al*, 2006). However, no such analyses exists for plant MATEs.

We report on the identification of amino acid residues responsible for the transport activity of TT12 in Chapter III of this dissertation.

AIMS OF THIS WORK

In general, members of the MATE family are involved in a multitude of transport functions across cell membranes. However, the plant MATES are poorly characterized and the identity of functionally crucial amino acids remains unknown. This work aimed to (i) use TT12 as a model plant MATE to identify crucial amino acids to its reported transport of C3G by a site directed mutagenesis approach, (ii) continue characterization of two novel putative MATEs from *Lupinus albus* (LaMATE1 and -2) implicated in the extrusion of citrate and isoflavonoids from roots (Tomasi *et al*, 2008) and (iii) identify novel MATE transporters in Arabidopsis, responsible for flavonoid transport.

We report:
(i) That heterologous expression of *LaMATE2* in yeast, resulted in yeast vesicles being able to transport genistein, which is amongst the other flavonoids, secreted from the roots of white lupin when P_i is limited (Weisskopf *et al*, 2006a).

(ii) That SDM of the TT12 protein revealed a single amino acid residue (E290A), that when mutated did not alter the reported tonoplast localization but completely abolished the transport function of C3G and failed to complement the PA deficient seed phenotype of the Arabidopsis *tt12* loss of function mutant.

(iii) The partial characterisation of three putative Arabidopsis flavonoid transporters (DTX 31, 35 and 28). *DTX31* transcripts were specifically up-regulated in Arabidopsis roots in response to salt stress. Furthermore, this up-regulation was found to be transient (maximum transcript abundance after 6h exposure to 75 mM NaCl) and correlated to a distinct alteration of flavonoids in root exudates from a loss of function mutant (*dtx31*). The *DTX31* promoter activity was found to correlate with this expression pattern, being most active only in the roots when transgenic plants (T2) transformed with a promoter::β-glucoronidase fusion were subjected to salt stress (150 mM NaCl). We suggest that DTX 31 may represent a plant MATE involved in the extrusion of secondary metabolites from roots during salt stress.

CHAPTER II: PHOSPHATE STARVATION INDUCES TWO MATE TRANSPORTERS IN WHITE LUPIN CLUSTER ROOTS: IMPLICATIONS FOR CITRATE AND ISOFLAVONOID SECRETION.

2.1. INTRODUCTION

White lupin (*Lupinus albus*, L.) is considered a major forage crop (Gladstones, 1970). White lupin grows on soils, with naturally limited inorganic phosphate, by producing special root structures (besides normal roots) called cluster roots. Immature and young, growing cluster roots excrete malate and low amounts of citrate while mature cluster roots excrete high amounts of carboxylates, mainly citrate (Fig. 2.1 A, C). The excretion of these compounds causes the acidification of the rhizosphere (Fig. 2.1 B) effectively mobilizing the soluble phosphorus in calcareous soils (Neumann and Romheld, 1999). A different situation occurs in acidic soils where dicarboxylates and tricarboxylates act as anion exchangers for rock-bound phosphate releasing phosphate from Fe-Al-P complexes (Ryan *et al*, 2001). Recent studies identified a citrate transport activity in protoplasts isolated from white lupin roots (Zhang *et al*, 2004).

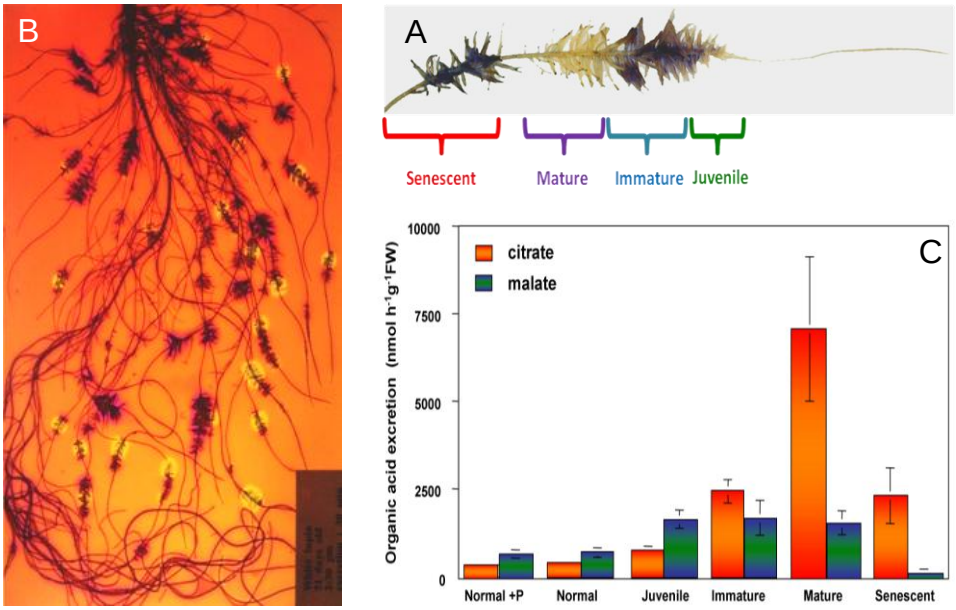


Figure 2.1. *Lupinus albus* cluster roots (A) are responsible for strong acidification of the rhizosphere (B) (Massoneau *et al*, 2001) and excrete large amounts of citrate in P-deficient conditions (C) (adapted from N.Tomasi, 2009).

The most abundant other secondary metabolites of leguminous plants are the isoflavonoids which occur in the shoots and roots (Ingham *et al*, 1983; Katagiri *et al*, 2000). The *Lupinus* genus is known to accumulate a wide range of isoflavonoids such as genistein and 2'-hydroxygenistein, which are secreted in higher amounts from cluster than from non-cluster roots and from P-deficient than from P-sufficient plants (Weisskopf *et al*, 2006).

These two isoflavones, which are accumulated as glucosides in the vacuole, may undergo further modifications by isopentenyl groups at different positions, yielding unique compounds e,g, luteone in white lupin (Tahara *et al*, 1989; Wojtaszek *et al*, 1993). It has been shown that flavonoids liberated from the roots of P-deficient white lupins are involved in P-acquisition directly by mobilizing soluble Fe-bound P and indirectly by limiting the microbial citrate mineralization and the activity of enzymes involved in microbial P acquisition (Tomasi *et al*, 2008).

Root exudates are often segregated into two classes of compounds: (i) low-molecular weight ones such as organic acids, amino acids, sugars, phenolics and (ii) high-molecular weight exudates, such as proteins and polysaccharides which comprise the larger proportion of root exudates (Walker *et al*, 2003). There are four different ways in which roots release their exudates from the living root system: (i) diffusion, (ii) ion channels, (iii) vesicle transport and (iv) ATP - dependent transport. The passive process involving gradients between the cytoplasm of intact root cells and the soil is used by roots to release low molecular weight hydrophobic organic compounds and was mainly discussed to occur for terpenoids and unglycosylated phenolics. In the typical cytosolic pH of approximately 7.1-7.4, low-molecular weight compounds weak acids and basis including amino acids are present as anions with low plasmalemma permeability and thus, they require other mechanisms of release.
In recent years, it has been shown that plant cells accumulate high concentrations of secondary metabolites in the intracellular bodies (Grotewold, 2001) which supports the idea of secondary compounds being transported via vesicle-like structures.

The phytochemicals might be also transported via ABC or MATE membrane transporters. Thus far, only plasma membrane localized ion channels have been shown to be involved in secretion of malate, citrate and oxalic acid in wheat, lupin and soybean respectively (Yan *et al*, 2002; Shen *et al*, 2005). A gene showing homology to MATEs was reported to be transcriptionally upregulated in white lupin subjected to P-deficiency (*LaMATE*, Uhde-Stone *et al*, 2005).

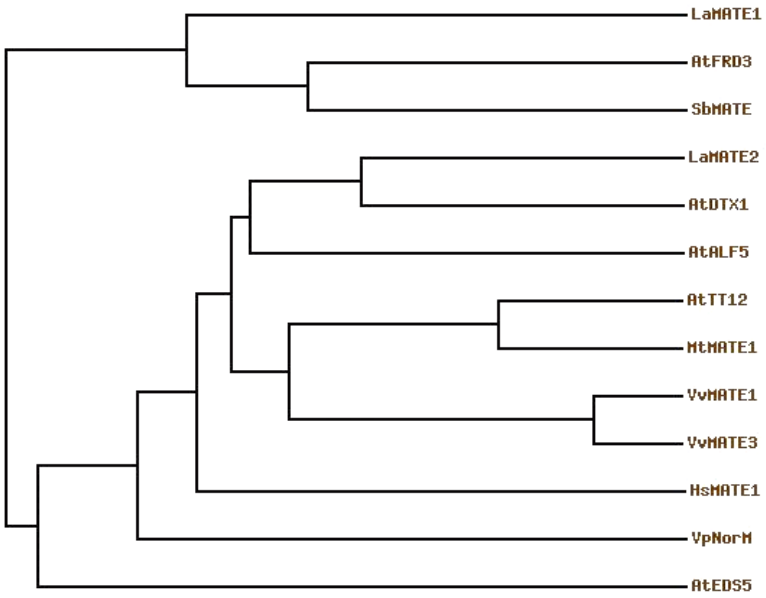


Figure 2.2. Phylogenetic analysis of functionally analyzed MATE transporters with LaMATE1and LaMATE2 (http://www.genebee.msu.su). The AGI codes or Genebank entries are as follows: AtFRD3, At3g08040; AtEDS5, At4g39030; AtTT12, At3g59030; AtDTX1, At2g04070; AtALF5, At3g23560; SbMATE1, ABS89149; HsMATE1, NP_060712; VpNorM, O82855, VvMATE1, GSVIVP00037700001; VvMATE3, GSVIVP00037696001; MtMATE1, CT485797_21.5. At, *A. thaliana*; Sb, *Sorghum bicolor;* Hs, *Homo sapiens*; Vp, *Vibrio parahaemolyticus*, Vv, *Vitia vinifera*, Mt, *Medicago truncatula.*

LaMATE (Uhde-Stone *et al*, 2005, here called *LaMATE1*) shows the highest amino acid sequence identity to Arabidopsis *FRD3* and *SbMATE1*, which are both responsible for excreting the citrate out of the cytosol (Durrett *et al*, 2007; Magalhaes *et al*, 2007) (Fig. 2.2). It has been demonstrated that excretion of carboxylates (i.e. citrate) might be also beneficial for iron (Fe) nutrition. In case of white lupins, it has been shown that cluster roots also occur under low iron availability and Fe^{3+} complexes with citrate. Knock-out mutants of the Arabidopsis MATE transporter *FRD3* (*ferric reductase defective 3*) showed reduced concentrations of citrate in the xylem (Durrett *et al*, 2007). In that study the accumulation of iron in the roots and leaves suggested that the iron translocation in the plant was disrupted in the absence of a functional FRD3. Sorghum MATE confers Al tolerance by a physiological process based on Al-activated citrate release into the rhizosphere (Magalhaes *et al*, 2007). These findings may suggest that the lupin MATE1 gene isolated under the phosphate starvation might be a possible candidate as organic acids transporter.

LaMATE2 show homology to MATE transporters mediating transport of flavonoids (*AtTT12*, *anthoMATE1* and *-3*, *MtMATE1*) (Fig. 2.2). This implicates that lupin MATE2 could be also involved in transport of phenolic compounds. Recent findings identified an Arabidopsis MATE transporter, the *Transparent Testa 12* (*TT12*) gene, to be involved in vacuolar deposition of proanthocyanidin

precursors (Debeaujon *et al*, 2001; Marinova *et al*, 2007). Similarly two genes from grapevine *anthoMATE1* and *anthoMATE3* were reported to be required for vacuolar transport of acylated anthocyanins (Gomez *et al*, 2009) whilst *Medicago MATE1* facilitates the vacuolar uptake of epicatechin 3'-*O*-glucoside for PAs biosynthesis (Zhao and Dixon, 2009).

Our interest in lupin lies in phosphate deficiency linked excretion of organic compounds by membrane transporters (Tomasi *et al* 2009, Santelia *et al* 2007, PhD thesis). Two cDNAs were previously isolated from white lupin grown under phosphate starvation (PhD thesis, Santelia, 2007). Both cDNAs show homology to plant MATEs and were designated *LaMATE1* and *-2*. Since both citrate and flavonoids have been implicated in phosphate utilisation strategies of white lupin, we investigated if LaMATE1 and -2 were able to transport these substances *in vitro.*

2.2. MATERIAL AND METHODS

2.2.1. CHEMICALS

^{14}C-citrate and ^{3}H-genistein were purchased from PhytoLab. For uptake experiments they were dissolved as a 0.1 µCi/ml stock in 50% ethanol.

2.2.2. STRAINS

The yeast strain YPH 499 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1) was used for heterologous expression of the *LaMATE1 and LaMATE2* cDNAs. The same strain was used for the isolation of microsomes.

2.2.3. PLASMID CONSTRUCTIONS

We constructed a 2μ yeast expression vector pNEV-Ura (Sauer and Stolz, 1994) containing the *LaMATE1* (cLM1) or *LaMATE2* (cLM2) cDNA by performing the amplification of cLM1 and cLM2 from genomic DNA isolated from white lupins under P deficiency with the primers LaMATE1_up (5' AAGGAAAAAAGCGGCCGCTACAACATGGCAGAGAATGGCACTGTGC 3') or LaMATE2_up (5'AAGGAAAAAAGCGGCCGCTACAACATGGAAGAGAGTCTAATAC CAAAACAGG 3'), respectively and LaMATE1_low (5' TTTTCCTTTTGCGGCCGCTTAGTT AATTAACACAGACATTGGGTGGCTTCTG 3') or LaMATE2_low (5' TTTTCCTTTT GCGGCCGCTTAGTTAATTAATGCTAGACTATTTTCTGCTGAAGATTC 3'), where NotI restriction sites are underlined. Obtained PCR products were inserted into the NotI restriction site of pNEV-Ura, resulting in pNEV_LaMATE1 and pNEV_LaMATE2, respectively. For both constructs,

PCR amplifications were carried out using the ExTaq (TAKARA) system. Lack of errors and correct incorporation was confirmed by sequencing.

2.2.4. TRANSFORMATION PROCEDURES

We performed yeast transformation following standard procedures (Gietz and Woods, 2002) and selected the transformants on minimal synthetic dropout medium lacking uracil (SD-Ura; 2% glucose, 1.7% Bacto-YNB w/o amino acids and $(NH_4)_2SO_4$, 1.4% amino acids mix, 0.5% $(NH_4)_2SO_4$, pH 5.5).

2.2.5. PREPARATION OF YEAST MEMBRANE VESICLES

Yeast membrane vesicles were isolated as previously described (Tommasini *et al*, 1996) with the modifications reported by Klein *et al* (2002). Briefly, transformed yeast strains were grown overnight in SD-Ura to an OD_{600} = 2-4, washed once with water and resuspended in YPD (Yeast Peptone-Dextrose; 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose). After that they were incubated for an additional 1 hour at 30°C with shaking. Cells were washed twice with water and resuspended to an OD_{600} =10 in lyticase buffer (1.1 M sorbit, 20 mM Tris-HCl pH 7.6, 1 mM dithiothreitol (DTT)) containing 57 units of lyticase per ml). After digestion (1.5-2 h), spheroblasts were collected by centrifugation (1200 x g, 10 min, RT). Cell pellets were then homogenized, on ice, in 30 ml of homogenization buffer [1.1 M glycerol, 50 mM Tris – ascorbate pH 7.4, 5 mM EDTA, 1 mM DTT, 1.5% (w/v) polyvinylpyrrolidone (PVP), 2 mg/ml bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer and 30 strokes with a tight-fitting glass piston. Two centrifugations (4000 x g, 10 min, 4°C) were used to remove unbroken cells and debris. The supernatants were collected, combined and centrifuged (100 000 x g, 45 min, 4ºC). The pellet containing microsomal membranes was resuspended to an OD_{600} = 4 in storage buffer (1.1 M glycerol, 50 mM Tris-MES pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF). Aliquots (250 µl) were immediately used for transport assays or stored at - 80ºC for further use.

2.2.6. IN VITRO TRANSPORT STUDIES

To study transport of substrates into membrane vesicles uptake experiments were performed with nitrocellulose filters (0.45-µm pore size; Millipore) using the rapid filtration technique (Tommasini *et al*, 1996). Thawed vesicles were mixed with transport buffer (0.4M glycerol, 0.1 M KCl, 20 mM Tris-MES, pH 7.4 and 1 mM DTT) in the ratio of 1:3 (v:v) in the presence of 0.1 mM citrate or 5µM genistein and in the absence or presence of 5 mM MgATP in a total reaction volume of 0.8 ml. At specified time points, aliquots (200 ul) were pipetted onto a prewet filter that was rapidly

washed twice with 3 ml of ice-cold transport buffer. Filters were air dried, immersed in 3 ml of scintillation cocktail (Ultima Gold™, Perkin Elmer, Inc.) and analysed using a liquid scintillation counter (Liquid Scintillation Analyzer, Tri-Carb 2900 TR, Packard).

2.3. RESULTS

2.3.1. *LaMATE1* EXPRESSED IN YEAST DO NOT SHOW CITRATE TRANSPORT ACTIVITY, HOWEVER *LaMATE2* ACTS AS A GENISTEIN TRANSPORTER

To investigate the transport activity of LaMATE1 and LaMATE2 biochemically, we expressed the *LaMATE1* and *LaMATE2* cDNAs in *Saccharomyces cerevisiae* and isolated total microsomal membrane vesicles for transport experiments. Physiological intactness of the microsomal fractions was verified by their capacity to accumulate ^{14}C-methylamine in the presence of MgATP due to acidification of the vesicle lumen. This method was used to confirm the intactness of the vesicles with each isolation of yeast microsomes.

Since our phylogenetic analyses suggested that *LaMATE1* might be involved in citrate transport (Fig. 2.2), we tested the ^{14}C-citrate uptake into the vesicles. Our results showed no time-dependent transport activity (Fig. 2.3) compared to the empty vector (pNEV only) control in the presence or absence of MgATP.

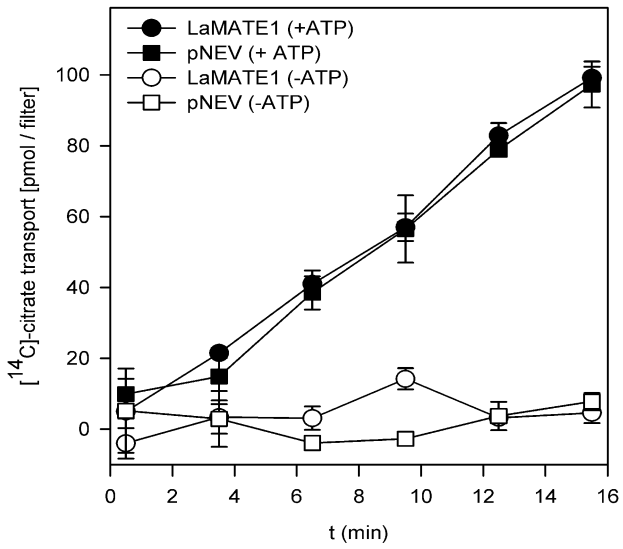


Figure 2.3. Uptake of radioactive citrate into microsomal vesicles isolated from yeasts transformed with an empty vector control (pNEV) and LaMATE1 in the presence or absence of MgATP as indicated.

LaMATE2 exhibits high homology to transporters suggested to act as flavonoid transporters (*AtTT12*, *VvMATE1*, *VvMATE2*, *MtMATE1*) (Fig. 2.2). We thus investigated the ability of LaMATE2 to transport ^{3}H-genistein, the isoflavonoid secreted from cluster roots into the rhizosphere.

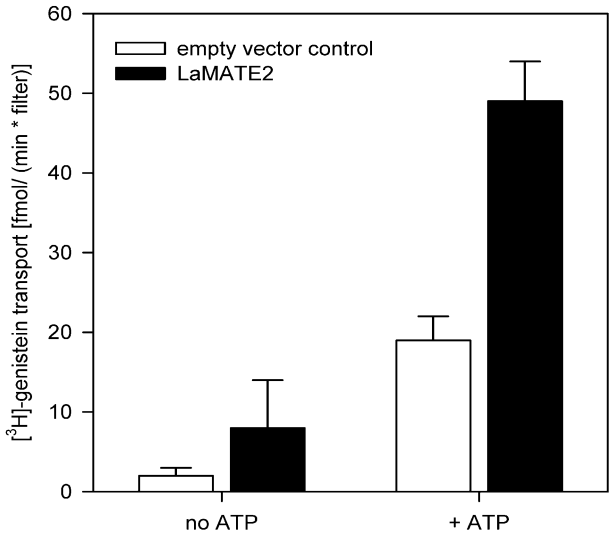


Figure 2.4. Uptake of radioactive genistein into microsomal vesicles isolated from yeasts transformed with an empty vector control (pNEV) and LaMATE2 in the presence or absence of MgATP.

Our results clearly showed that *LaMATE2* expressed in yeast acts as a genistein transporter in the presence of MgATP compared to the empty vector control (Fig. 2.4).

2.3. DISCUSSION

The role of *Lupinus albus* cluster roots in the physiology of phosphorus acquisition and excretion of organic acids and isoflavonoids in response to P deficiency has been shown in recent studies (Neumann *et al*, 2000; Uhde-Stone *et al*, 2003; Penaloza *et al*, 2005; Tomasi *et al*, 2009). However, very little is known about the transporters involved in these processes.

Soil properties and microbial activity control the availability of soil phosphorus (Brady, 1990). White lupin, by forming cluster roots and releasing high amounts of citrate can increase P accessibility (Shen *et al*, 2003; Shane and Lambers, 2005). Under the P starvation, white lupin also releases large amounts of mostly genistein- and hydroxygenistein-derivated flavonoids (Neumann *et al*, 2000; Weisskopf *et al*, 2006). Tomasi *et al* (2008) have confirmed that plant-secreted flavonoids can mobilize inorganic phosphorus and decrease soil microbial respiration, citrate mineralization and soil phosphohydrolase activities without decreasing the amount of ATP in the soil. It has also been reported that liberation of flavonoids into the rhizosphere plays an important role in its efficient P-

acquisition strategy by solubilising Fe-bound P and by preventing the microbial mineralization of citrate.

Initially, it was demonstrated that white lupin proteoid roots express a putative MATE transporter when subjected to P deficiency (Uhde-Stone *et al*, 2003). The expression induction timing was also consistent with the theory that MATE transporters in white lupin might be involved in citrate and malate excretion. Further investigation demonstrated that this MATE was also expressed under –Fe, -N, -Mn and +Al stresses (Uhde-Stone *et al*, 2005). It has been also shown that it is most probably localised to plasma membrane.

Using a cDNA-AFLP approach, two cDNAs designated *LaMATE1* and *LaMATE2* were isolated from the juvenile, mature and senescent roots of white lupin grown under P deficient conditions. The *LaMATE1* sequence was consistent with the lupin MATE previously described (Uhde-Stone *et al* 2003). Both *LaMATE1* and *LaMATE2* were specifically induced upon P deficiency mostly in the mature roots, where solubilisation, soil extraction and uptake of phosphate into the plant occur. *LaMATE1* displays homology to *FRD3* in Arabidopsis and *SbMATE* in sorghum. *FRD3* is involved in the excretion of citrate into the xylem and is necessary for long-distance iron transport to the leaves (Rogers *et al*, 2002; Green and Rogers, 2004; Durett *et al*, 2007). *SbMATE* is an aluminium-activated citrate transporter responsible for the aluminium tolerance of *Sorghum bicolour* via the Alt_{SB} locus (Magalhaes *et al*, 2007). Our phylogenetic analyses and previous reports strongly suggest LaMATE1 playing a role in citrate exudation, however we were unable to demonstrate any citrate transport activity in yeast vesicles expressing the LaMATE1 cDNA. Sequence comparisons may not always be indicative of gene function and our results seem to confirm that LaMATE1 is not a citrate transporter since *LaMATE1* was also unable to complement the *frd3* mutant (Uhde-Stone *et al*, 2005). Alternatively, *LaMATE1* may not be expressed or not functional in yeast. Since our construct does not contain a tag, we were not able to verify this hypothesis.

LaMATE2 shows the highest homology to *TT12* (Arabidopsis), *anthoMATE1* and *3* (*Vitis vinifera)* and *MATE1* (*Medicago truncatula)*. All of these transporters are involved in flavonoid transport. *AtTT12* is a vacuolar anthocyanin/H^+-antiporter required for the vacuolar accumulation of proanthocyanidin precursors in the seed (Marinova *et al*, 2007). In grapevine, anthoMATE1 and anthoMATE3 are responsible for H^+-dependent transport of acylated anthocyanins into the vacuole (Gomez *et al*, 2009) and in *Medicago truncatula MATE1* mediates uptake of epicatechin 3'-*O*-glucoside for biosynthesis of proanthocyanidins. *MtMATE1* was also able to complement the seed proanthocyanidin phenotype of the Arabidopsis *tt12* mutant (Zhao and Dixon, 2009). Since roots of white lupin produce only isolflavonoid, it is tempting to speculate that LaMATE2 might be involved in isoflavonoid transport. We expressed *LaMATE2* in yeast and performed transport activity analysis. Our data suggest that LaMATE2 acts as a genistein transporter in the presence of MgATP (Fig. 2.4) and therefore demonstrate that it may encode an isoflavonoid transporter.

The results we obtained, together with the preliminary findings of a previous doctoral thesis (Santelia, 2007) support the hypothesis that the *LaMATE2* transporter mediates the excretion of isoflavonoids from white lupin cluster roots during P deficiency.

Future studies on LaMATE2 should include the subcellular localisation and analysis whether genistein export across cluster root plasma membrane is proton dependent. Furthermore it would be interesting to transform LaMATE2, under control of a root specific promoter, into Arabidopsis to analyse if the roots of transgenic plants are then also able to secrete flavonoids. It should be taken into account that we were unable to repeat our transport experiments more than twice, for that reason future work should also contain further transport experiment analysis.

CHAPTER III: CHARACTERISATION OF MUTANTS OF THE ARABIDOPSIS FLAVONOID/H^+ MATE TRANSPORTER *TRANSPARENT TESTA12* IDENTIFIES CONSERVED AMINO ACIDS CRITICAL FOR TRANSPORT AND VACUOLAR TARGETING.

Anna Polinceusz, Katharina Schmid, Barbara Weder, Jean-Marc Routaboul, Enrico Martinoia, Markus Klein

To be submitted to JBC

3.1. ABSTRACT

Multidrug and toxic extrusion (MATE) proteins are membrane transporters presented in bacteria, plants and animals. Whilst model bacterial and human MATEs have been characterized, in detail, little is known about structure-function relationships of plant MATEs. As a model plant MATE we utilized the well characterized Transparent Testa12 (TT12) from Arabidopsis, recently identified as a flavonoid/H^+-antiporter able to transport glucosylated anthocyanin and proanthocyanidin precursors. After extensive amino acid sequence alignments and analyses, using all 56 Arabidopsis MATEs and other well characterized MATEs from bacteria and humans we identified 8 amino acids universally conserved and 6 conserved only between the Arabidopsis MATEs. Using site-directed mutagenesis, we systematically altered these amino acids in TT12. We analysed the effect of these mutations by transforming the Arabidopsis tt12 loss-of-function mutant and specifically looked for mutations which failed to complement the marked proanthocyanidin deficient seed coat characteristic for tt12. Several conserved glycines were impotant for correct trafficking of TT12 to the tonoplast. Only one single amino acid residue E290, when mutated, still localized to the tonoplast (as the native TT12) but failed to rescue the PA deficient seed coat phenotype of the tt12 mutant. The flavonoid profile of seeds using LC-MS, was comparable in tt12 and in tt12 seeds expressing TT12E290A under control of the BANYULS5 promoter. When TT12E290A was heterologously expressed in yeast, vesicles were unable to transport cyanidin-3-glucoside, confirming that the E290 is critical to the proper functioning of TT12. We speculate that E290 is involved in cation binding in TT12.

3.2. INTRODUCTION

Plants are known to generate a great number of secondary metabolites which are categorized into several categories according to their chemical structure and their biosynthetic pathways. Of these groups, flavonoids represent one class of phenolic compounds that play important physiological roles in plants including sexual reproduction, seed dispersal, defense or germination (Burbulis *et al*, 1996; Harborne *et al*, 2000). Flavonoids possess diverse chemical structures but share a universal C6-C3-C6 core. Major classes of flavonoids include flavonols or flavones (yellow color), anthocyanins (red to purple) and proanthocyanidins (PA). In Arabidopsis, PAs are condensation products of flavan-3-ol units (Xie *et al*, 2003) and are only produced at early stage of seed development, where they accumulate in the seed coat (Debeaujon *et al*, 2003). During seed maturation the pigments confer a brown color due to oxidation (Devic *et al*, 1999; Debeaujon *et al*, 2001).

The Arabidopsis *TT12* gene (*AtDTX41*/At3G59030) was identified as a transporter-like membrane protein involved in biosynthesis of flavonoids (Debeaujon *et al*, 2001). In that study, seeds from loss-of-function mutants were shown to lack accumulation of both PA polymers and epicatechin precursors in vacuoles of the seed coat endothelium. Further characterisation demonstrated that TT12 localised to the tonoplast and when heterologously expressed in yeast, transports glucosylated anthocyanin and epicatechin but not glycosylated flavonols or procyanidin dimers in the presence of ATP (Marinova *et al*, 2007; Zhao and Dixon, 2009).

The Arabidopsis genome contains 56 genes coding for putative MATE transporters (Multidrug And Toxic Compound Extrusion) (Li *et al*, 2002; Rogers *et al*, 2002). At least six of these genes have been partially characterised and found to be involved in diverse physiological roles including (i) lateral root formation (ALF5/*AtDTX19*) (Diener *et al*, 2001), (ii) iron homeostasis (FRD3/*At DTX43*) (Rogers *et al*, 2002), (iii) disease resistance (EDS5/*At DTX47*) (Nawrath *et al*, 2002), (iv) multidrug and heavy metal detoxification (*AtDTX1*) (Li *et al*, 2002), (v) anther dehiscence and pollen development (FFT/*AtDTX35*)(Thompson *et al*, 2010) and (vi) biosynthesis/transport of PAs (TT12/ *AtDTX41*)(Debeaujon *et al*, 2001; Marinova *et al*, 2007).

MATE transporters which are distributed in all kingdoms of living organisms constitute the most recently discovered family of multidrug transporters acting through H^+ or Na^+ exchange (Omote *et al*, 2006). The first MATE (NorM) was identified from the bacterium *Vibrio parahaemolyticus* and shown to mediate norfloxacin resistance when heterologously expressed in *E. coli* (Morita *et al*, 1998). The human MATEs *hMATE1* and *2-K* were more recently described (Otsuka *et al*, 2005). hMATE1 is a polyspecific organic cation exporter, transporting toxic cations such as tetraethylammonium into urine and bile. Three conserved acidic amino acid residues, in a predicted transmembrane region of NorM were demonstrated to be crucial for the Na^+ -driven drug transport process (Otsuka *et al*, 2005). Likewise, a glutamate in transmembrane helix 7, E273 in hMATE1 and E251 of NorM from *Vibrio parahaemolyticus*, are essential for transporter function (Otsuka *et al*, 2005). In the recent X-ray structure of NorM from *Vibrio cholerae*, NorM reveals an outward-facing conformation with holes facing the extracellular space and a total of 12 transmembrane helices. In this stage, which is suggested to represent a conformation with high affinity for the cation but not for the substrate, the glutamate in helix 7 is part of the cation binding site in the pore (He *et al*, 2010). Interestingly, alignment of NorM and hMATE1 with TT12 revealed that this residue was conserved in TT12 (E290). Histidine and cysteine residues were shown to be essential to the H^+/organic cation antiport activity in rat kidneys. These residues (H385, C62, C126) play an important role in human MATE1 and MATE2-K and it has been suggested that the cysteine residues may act as substrate-recognition sites (Asaka *et al*, 2007).

Plant MATEs have, to our knowledge, not been extensively analysed to determine functional catalytic residues. In this work, we have performed amino acid sequence alignments of all 56 Arabidopsis MATEs and included prokaryotic and human MATEs. A total of 6 amino acid residues were conserved in all MATE proteins of the Arabidopsis. As a first step towards the functional dissection of plant MATE transporters, 14 conserved residues in TT12 were altered by site-directed mutagenesis (SDM). Interestingly, 6 of the conserved amino acids were not affecting the ability of the mutated TT12 version to complement the *tt12* PA deficiency in the seed coat suggesting that they are not necessary for function. In contrast, several strongly conserved cytosolic glycines appear to be important for correct targeting of TT12 to the vacuolar membrane. The mutation of the conserved glutamate in helix 7 (E290A) failed to functionally rescue the PA deficient seed coat phenotype of the *tt12* mutant, despite correct localisation to the vacuole. When performing proton pumping experiments using vesicles isolated from yeast heterologously expressing native $TT12_{WT}$ or mutated $TT12_{E290A}$, the cyanidin 3-glucoside (C3G) dependent reduction in proton pumping activity mediated by $TT12_{WT}$ was absent when $TT12_{E290A}$, was expressed suggesting that the E290 is critical for C3G/H^+-antiport activity.

3.3. METHODS

3.3.1. STRAINS, CHEMICALS AND PLANT MATERIAL

Cyanidin 3-glucoside (C3G) was purchased from Extrasynthèse. For uptake experiments C3G was dissolved as a 0.1 M stock in 50% methanol/0.1.% HCl.

The yeast strain YPH 499 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1) was used for heterologous expression of the *TT12*, $TT12_{E290A}$ cDNA and *TT12* derivatives. The same strain was used for the isolation of microsomal membrane vesicles.

The *Arabidopsis thaliana tt12* mutant was isolated from a collection of T-DNA transformants in the Ws-1 background (Errampalli *et al*, 1991).

3.3.2. TRANSMEMBRANE TOPOLOGY PREDICTION AND SEQUENCE ALIGNMENT

Alignment and transmembrane (TM) topology predictions of *A. thaliana* MATE proteins were acquired from the ARAMEMNON database (Schwacke *et al*, 2003).
NCBI accessions of AtTT12 MATE transporter orthologs used for the alignment: NorM from *V. cholerae* (AAF94694.1), NorM from *V. parahaemolyticus* (BAA31456.1), Nt-JAT1 from *Nicotiana*

tabacum (CAQ51477.1), MatE from *Dictyostelium discoideum AX4* (XP_642174.1), SCL47A from *Homo sapiens* (NP_060712.2).

Transmembrane helices topology prediction was performed using HmmTop_v2 (http://www.enzim.hu/hmmtop/server/hmmtop.cgi). Multiple sequence alignement was performed with ClustalW alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

3.3.3. SITE-DIRECTED MUTAGENESIS, PLASMID CONSTRUCTION AND TRANSFORMATION PROCEDURES

Point mutations were introduced into pDONR207::$TT12_{WT}$ (Curtis and Grossniklaus, 2003) using the QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's guidelines. To modify $TT12_{wt}$ to $TT12_{E290A}$, the following primer pair was used (forward: 5' GCAG TTATGCTATGTTTGGCGATATGGTACAACCAAGGGC 3', reverse: 5' GCCCTTGGTTGTACC ATATCGCCAAACATAGCATAACTGC 3'). Other amino acid residues were substituted with the primers shown in the Supplemental Table 1. The PCR products were recombined into the modified binary destination vector $pMDC83_{pBAN5}$ (Curtis and Grossniklaus, 2003) carrying the BANYULS 5 promoter (16) driving seed-coat specific expression of genes added in between the *attL* sites for *tt12* rescue studies or pEarleyGate104 (Earley *et al*, 2006) for localization using a conventional LR clonase reaction (Invitrogen™ Gateway®). Final constructs were introduced into the *Agrobacterium tumefaciens* GV3101 strain by electroporation, using a Genepulser (2.5 kV; 100Ω; 25 µF, Bio-Rad, Reinach, Switzerland)**.** Stable transformation of *Arabidopsis* plants and transient expression in *Nicotiana benthamiana* leaves using infilitration was performed as previously described (Nesi *et al*, 2000; Voinnet *et al*, 2003; Walter *et al*, 2004).

3.3.4. GENE EXPRESSION ANALYSIS

Total RNA was extracted from leaves using the Plant RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland). Reverse transcription was carried out from each sample from 1 µg of purified RNA using the SuperScript II RT Kit (Life Technologies). PCR amplifications were performed from 100 - 200ng of cDNA using the Expand High Fidelity PCR system (Roche). Accuracy of the sequences was verified by sequencing.

3.3.5. LC/MS ANALYSES OF SEED FLAVONOIDS

Flavonoids from mature seeds were extracted and analyzed as described previously (Routaboul *et al*, 2006).

3.3.6. PREPARATION OF YEAST MEMBRANE VESICLES AND IN VITRO TRANSPORT STUDIES

Yeast membrane vesicles were isolated from spheroblasts following described methods (Tommasini *et al*, 1996; Klein *et al*, 2002). Proton translocation across the vesicle membranes was determined by measuring the quenching and recovery of fluorescence of the ΔpH probe 9-amino-6-chloro-2-methoxyacridine (ACMA) (Gomez *et al*, 2009). The assay mixture contained transport buffer (0.4 M glycerol, 0.1 M KCl, 20 mM Tris-MES, 1 mM dithiothreitol, 6 mM $MgCl_2$, pH 7.4), 2 µM ACMA, 300 µg/mL of yeast microsomes (corresponding to of total protein) and assays were performed in the presence or absence of 0.2 mM C3G. Fluorescence intensities of the probes were measured using a plate reader spectrophotometer (Fusion™ α, Packard) at a wavelength of 485 nm, after excitation at 425 nm. After stabilization, proton pumping was started by addition of 5 mM MgATP. When reaching steady state, the pH gradient was abolished by addition of $(NH_4)_2SO_4$ to a final concentration of 5 mM.

3.3.6. SUBCELLULAR LOCALISATION AND CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

Subcellular localisation of GFP and YFP fusions of the SDM TT12 versions (with the exception of TT12 versions with G416 mutations to I and Q) was examined by transient expression in *N. benthamiana* leaves and, after stable transformation of *Arabidopsis*, using CLSM. Transient expression of leaves using infiltration of *A. tumefaciens* GV3101 transformed with corresponding plasmids was preformed as described previously (Voinnet *et al*, 2003; Walter *et al*, 2004). CLSM analyses were conducted after incubating *N. benthamiana* plants for 48 hours at 25ºC. Epidermal peels from infiltrated *N. benthamiana* leaves were prepared as described (Frangne *et al*, 2002). Roots of sterile-grown transgenic *tt12* and $tt12_{E290A}$ plants ectopically expressing *GFP5-cTT12* or *GFP5-*$cTT12_{E290A}$ were analyzed as previously described (Marinova *et al*, 2007). Confocal laser scanning microscopy (TCS SP2-x1 full-spectrum confocal microscope attached to Leica DM IRE2 inverted fluorescence microscope) was used to capture single optical sections. GFP/YFP were excited with a 488-nm Ar and a 543-nm HeNe laser and fluorescence emission images averaged over eight frames were captured in independent channels.

3.3.7. WHOLE MOUNT VANILLIN STAINING OF SEEDS

Immature seeds (4 DAF) from 10 stably transformed Arabidopsis lines (T2) transformed with pMDC83Ban5pr-TT12$_{mut}$ constructs were stained in a solution of 1% (w/v) vanillin and 6 N HCl at room temperature for 5 min to detect the presence of PAs, as previously described (Aastrup *et al*, 1984; Debeaujon *et al*, 2000). Vanillin changes color to red upon binding to flavan-3, 4-diols, flavan-4-ols and flavan-3-ols when present either as monomers or as terminal subunits of PAs (Desphande *et al*, 1986). Bright field images of vanillin-treated seeds were captured using the Leica DM R microscope.

3.3.8. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Flavonols were extracted with acetone: methanol: water: TFA (40:32:28:0.05; v:v) and measured by LC-MS and proanthocyanidin after acid catalyzed hydrolysis (Routaboul *et al*, 2006).

3.4. RESULTS

3.4.1. MULTIPLE ALIGNMENT OF PROTEIN SEQUENCES OF TT12 AND VARIOUS MATES REVEALED CONSERVED AMINO ACID RESIDUES

The predicted amino acid sequences of all putative Arabidopsis MATEs (AtDTX1 to AtDTX56) were aligned with those of NorMI and NorMII from *Brucella melitensis*, the recently crystallized NorM from *Vibrio cholerae* (He *et al*, 2010), NorM from *Vibrio parahaemolyticus*, PmpM from *Pseudomonas aeruginosa*, human MATEs and the tobacco nicotine transporter JAT1 (Morita *et al*, 2009). A subset of these alignements is depicted in Fig. 1. a total of 5 'boxes' were identified which included 'clusters' of conserved amino acids either in all Arabidopsis or in all MATEs transporters chosen (Fig. 1; Suppl. Fig. 1). The amino acids T115, Q198 and C317 in TT12 were found to be conserved in the Arabidopsis MATEs but not in the bacterial MATEs and in human MATE2, additionally amino acids S336 and Y436 were found to be conserved in the Arabidopsis MATEs but not in the bacterial MATEs. Thus, all of these amino acid could indicate specificity for plant MATE transporters. On the contrary, the amino acids G108, Q119, G229, E290, G232, G416, G420, G456 and W458 in TT12 were conserved throughout all aligned MATE protein sequences. Strikingly, a total of 6 glycines are conserved. We systematically generated site-directed mutations in all of these conserved amino acids by replacing T115, Q119, Q198, E290, C317, S336, Y436 and W458 with alanine. In the case of the glycines (G108, G229, G232, G416, G420, G456) they were first replaced

by the bulky, hydrophobic isoleucine residue. For more refined analysis, glycines were also exchanged with alanine and/or glutamine representing slightly more hydrophobic and more bulky but polar side chains, respectively. All mutant *TT12* versions were expressed under control of the PA-specific *BANYULS5* promoter (Devic *et al*, 1999) in the *tt12* background to investigate transporter functionality in T2 seeds. Histochemical vanillin staining of immature seeds was used to classify the investigated conserved amino acids into groups: either the mutated TT12 complemented the *tt12* seed chemotype to wild type, thus exhibiting the typical red vanillin staining indicative of PA precursor presence, or the seeds exhibited only the faint rose vanillin staining typical for *tt12* suggesting that the corresponding mutation could not rescue *tt12* (Suppl. Fig. 2). In order to differentiate between $TT12_{mut}$ versions that were not able to complement due to loss of transport function or due to incorrect targeting of the transporter (not to the vacuolar membrane), mutant TT12 versions were localized as GFP or YFP fusions (with exception of G416 and W458) in transient expression experiments (Suppl. Fig. 3). Taken together, the amino acids chosen for site-directed mutagenesis were broadly classified (Fig. 2). Q119A, Q198A, C317A, S336A, Y436A mutations did not affect TT12 function and localization and likewise also $TT12_{W458A}$ fully rescued the *tt12* seed phenotype. In the case of the conserved glycine residues, G to I, A or Q replacement lead to different levels of *tt12* complementation that went along with varying degrees of correct *vs* incorrect subcellular targeting. $TT12_{G108I,\ G420I,\ G420Q,\ G420A}$ exhibited partial localisation to the tonoplast and TT12 $_{G229I,\ G229A}$ localised to the other membranes. As a general rule, exchanging polar G with the bulky hydrophobic I residue lead to complete mistargeting of TT12. T115A situated in the first cytosolic loop exhibited partial *tt12* complementation and was correctly targeted. As only the E290A mutation in transmembrane helix 7 lead to an apparent complete loss of function phenotype (no *tt12* complementation, correct localisation), it was investigated in more depth.

3.4.2. $TT12_{E290A}$ IS UNABLE TO FUNCTIONALLY COMPLEMENT THE PA DEFICIENT SEED PHENOTYPE OF *TT12*

The Arabidopsis loss-of-function mutant *tt12* (4), was transformed with a *TT12* wild type gene and the SDM version (E290A), both under the control of the BANYULS5 promoter (Pro_{Ban5}*::*$TT12_{wt}$*/tt12* and Pro_{Ban5}*::*$TT12_{E290A}$*/tt12*, respectively). Expression of these constructs was verified, by sqRT-PCR, in the seeds of immature siliques from T2 transgenic plants. *TT12* transcripts were present in the seeds of wild-type (Ws-2), $TT12_{wt}$*/tt12* and $TT12_{E290A}$*/tt12* plants but not in the seeds of *tt12* plants (Fig. 3A).

The ability of TT12 to deposit PAs in the mature seed coat was analysed using DMACA (*p*-dimethylaminocinnamaldehyde) staining (Debeaujon *et al*, 2000). Whilst seeds of wild-type and $TT12_{wt}$*/tt12* plants stained to a dark color (indicative of PAs), the seeds of $TT12_{E290A}$*/tt12* plants

(verified by RT-PCR, Fig. 3A) stained comparably to the seeds of *tt12* plants (indicative of PA absence, Fig. 3B). We further, performed vanillin staining to confirm PA accumulation in immature T2 seeds (torpedo stage of embryo development). Similarly to the DMACA staining, the seeds of $TT12_{E290A}$/*tt12* plants contained no PAs, comparable to *tt12* seeds (Fig. 3C).

3.4.3. SUBCELLULAR LOCALISATION OF $TT12_{E290A}$ IS IDENTICAL TO THE $TT12_{WT}$

The subcellular localisation of the $TT12_{E290A}$ and wild-type TT12 was examined by laser confocal laser scanning microscopy (CLSM) of YFP or GFP fusions detected in root hairs (Fig. 3A) or root tip cells (Fig. 4B, C) of *Arabidopsis* (Ws-2) stably transformed with these reporter constructs. As a control, cell walls of root cells were counterstained with 50 µM propidium iodide as previously described (Marinova *et al*, 2007) (Fig. 4C). Evidently, and as described for the transient expression experiments, the E290A mutation does not affect TT12 targeting to the vacuolar membrane.

3.4.4. $TT12_{E290A}$/*TT12* AND *TT12* SEEDS HAVE THE SAME FLAVONOID COMPOSITION

Immature seeds of *tt12* exhibit a deficiency in the accumulation of PA precursors and PA polymers that are synthesized in the innermost integument, in some cells of the chalazal tissue and the micropyle (Debeaujon *et al*, 2003). Apart from PAs, also other flavonoid compounds are synthesized in a cell-specific manner during seed development (Routaboul *et al*, 2006; Kleindt *et al*, 2010). To test if SDM variants of TT12 altered the flavonoid composition in seeds, a liquid chromatography-mass spectrometry (LC-MS) approach was used.

Flavonoid extracts and pellet remaining after acid-catalyzed hydrolysis were separated into soluble and insoluble fractions. A small amount of PAs were detected in the insoluble fraction of *tt12* and $TT12_{E290A}$/*tt12* seeds when compared to Ws-2 seeds and none occurred in the soluble fraction (Fig. 5).

Furthermore, the amounts of quercetin-3-*O*-rhamoside (Q3-OR) and biflavonols were also reduced to ~70 and 40 % in *tt12* and $TT12_{E290A}$/*tt12*, respectively, when compared to Ws-2 or $TT12_{wt}$/*tt12*. Besides Q3-OR, quercetin-3,7-di-O-rhamnoside and kaempferol-3,7-di-O-rhamnoside the quantities of all other flavonoids were not significantly affected by the E290A mutation (Fig. 5).

3.4.5. THE E290A MUTATION AFFECTS THE TRANSPORT ACTIVITY OF TT12

To verify whether the transport activity of TT12 SDM variants was comparable to the $TT12_{wt}$, $TT12_{E290A}$ and $TT12_{wt}$ were cloned into the pNEV-Ura vector (Sauer and Stolz, 1994) and expressed in *Saccharomyces cerevisiae*. Prior to transport experiments with yeast vesicles, the presence of the heterologous $TT12_{wt}$ and $TT12_{E290A}$ was verified by Western blot and localisation studies (Suppl. Fig.

4). Using the H^+ sensitive dye 9-amino-6-chloro-2-methoxyacridine (ACMA) we were able to (i) determine the physiological intactness of yeast vesicles as previously described (Gomez *et al*, 2009) and (ii) demonstrate that the addition of cyanidin-3-glucoside reduced H^+ pumping into yeast vesicles expressing $TT12_{wt}$ (Fig. 6B). Conversely this did not occur in yeast vesicles expressing $TT12_{E290A}$ (Fig. 6C), comparable to the vector control (Fig. 6A), suggesting that $TT12_{E290A}$ is expressed as a non-functional protein in yeast.

3.5. DISCUSSION

The MATE family has been described as a membrane protein family occurring ubiquitously across the taxonomic kingdoms (Brown *et al*, 1999). Typically, they are involved in a multitude of transport functions across membranes (Yazaki *et al*, 2008). However, the identification of conserved amino acid signatures or domains essential to this transport function remains poorly characterised, with no reports for plant MATEs.

We compared the amino acid sequence of the Arabidopsis flavonoid MATE transporter, TT12 (Marinova *et al*, 2007) against the 55 putative Arabidopsis MATEs in addition to other MATEs where amino acids critical to their transport function were described (NorM and hMATE1). Interestingly, TT12 shares no cytosolic N- or C-terminal amino acids characteristic of the protein sequences of the 55 Arabidopsis MATEs, NorM and hMATE1. However, a number of amino acids were conserved between TT12 and the other MATEs, including E290 positioned in transmembrane 7 of TT12. A total of five boxes were detected which include conserved amino acids (Fig.1) that may guide sequence-based identification of MATE transporters.

We took advantage of the easily scorable phenotype of *tt12* seeds indicative of absence of PA biosynthesis due to lack of transporter function and localization experiments to classify conserved amino acids (Fig. 2). Interestingly, a large number of these residues (Q119, Q198, C317, S336, Y436, W458, partially T115) are not critical for TT12 function as demonstrated by rescuing the *tt12* seed phenotype and in most cases by localization experiments. This raises the question of the biological significance of their evolutionary conservation.

Six conserved glycine residues were detected, four of which were found in plant, mammalian and bacterial MATE proteins. Whenever these glycines were replaced with the bulky and hydrophobic isoleucine, *tt12* complementation was absent or only partial. At the same time, the protein was no longer correctly targeted to the vacuolar membrane (Fig. 2; Suppl. Fig.3). In contrast, glycine replacement with glutamine or alanine produced less drastic phenotypes with usually partial *tt12* PA rescue and TT12 localization similar to the wild-type protein or resulting in a mixture where fluorescence was detected on the tonoplast and on further endomembranes. This analysis is in accordance with the hypothesis that small, hydrophilic residues at the glycine positions (108, 229,

232, 416, 420, 456) render conformational flexibility to the overall protein structure which is critical for correct trafficking of TT12 to the vacuolar membrane.

The survey of all mutations introduced into TT12 lead to only one residue, E290 in predicted transmembrane helix 7, the mutation of which lead to a clear loss-of-function phenotype. The $TT12_{E290A}$ version failed to complement *tt12*, localized correctly and lost the ability to perform flavonoid/H^+ antiport activity as shown by lack of effect of C3G addition in a proton-pumping assay.

A glutamate residue in transmembrane helix 7 is conserved in all MATE transporters. Further, mutation of the corresponding glutamates in the bacterial NorM transporters as well as in human MATE1 led to dysfunctional proteins. The recently published structure of the NorM-VC protein from *Vibrio cholerae* representing a MATE transporter in its high-affinity cation-binding state shows that the cation-binding site of NorM-VC is formed by residues from TM7, TM8 and TM10–TM12, including E255 (E290 in TT12) (He *et al*, 2010). As an acidic residue, E255 (NorM-VC) faces the internal cavity and is in proximity to the cavity-located cation. Thus, our results are in accordance with the hypothesis that E290 in TT12 exerts a cation-binding function similar to E255 in NorM.

Apart from E255, two further acidic residues in NorM-VC, D36 (TM1) and D371 (TM10), are predicted to be critical for cation binding as supported by mutational studies of the corresponding aspartates in *V. parahaemolyticus* (Otsuka *et al*, 2005). Interestingly, these aspartates and also further amino acids forming the cation binding site in NorM-VC are not fully conserved in TT12 with the exception of Y436 in TM11 of TT12 (Fig. 1). Y436 is not critical for TT12 function as seen in the seed rescue screen. It is tempting to speculate that the cation-binding site is composed of different amino acids when protons instead of Na^+ is exchanged but needs a glutamate in TM7 irrespective of the nature of the cation. Our failure to detect any further TT12 version with an effect on the PA precursor transport function – apart from E290 - suggests that none of the conserved residues we identified are critical for the binding of the flavonoid substrate.

FIGURE LEGENDS

Figure 1. Amino acid sequence alignement of AtTT12 with selected MATE transporter orthologs (see Experimental section for NCBI accessions). Identical residues are shaded yellow. The extra C-terminal sequences of the slime mold and human MATEs are not comparable to the other transporters and were omitted for clarity. The HmmTop_v2-based prediction of transmembrane helices (TM) in TT12 is marked by blue bars. The TMs of the crystallized NorM from *Vibrio cholerae* (NorM_Vc; Protein Data Bank 3MKT and 3MKU; He *et al*, 2010) and the residues constituting the cation-binding site in the pore are highlighted by blue letters and red boxes, respectively. Amino acids chosen for mutagenesis based on conservation in all Arabidopsis MATE only or conserved in all pro- and eukaryotic MATE transporters chosen for alignement are highlighted in green and pink, respectively. Alignement of all 56 Arabidopsis MATE proteins resulted in five short stretches containing conserved amino acids which are highlighted by red bars below the alignement and noted as Boxes 1-5. An optical presentation of the conservation observed in these boxes is given in Suppl. Figure 1.

Figure 2. Summary of effects of mutations in conserved TT12 residues. (A) Representative examples of vanillin-staining results demonstrating PA presence in WT and in $TT12_{C317A}$/*tt12* immature seeds while *tt12* and $TT12_{G420I}$/*tt12* immature seeds lack PAs. Bars = 50 µm. (B) Representative $TT12_{mut}$-YFP localization examples. Upper panel: Tonoplast localization of $TT12_{S336A}$-YFP. Bar = 50 µm. Lower Panel: Localization of $TT12_{G420I}$-YFP to endomembranes. Bar = 10µm. Depicted are *N. benthamiana* protoplasts isolated from Agrobacterium-infiltrated leaves. Left panels are merged CLSM images where YFP is false-colored in green and chlorophyll auto-fluorescence is red. Right panel: Corresponding DIC images. (C) Table summarizing all $TT12_{mut}$/*tt12* complementation and $TT12_{mut}$-YFP localization information. N.d., not determined. n.d. = not determined; n.l. = localisation failed

Figure 3. $TT12_{E290A}$ expressed under the control of the *BANYLUS5* promoter does not complement *tt12*. (A) RT-PCR. (B) Dry seeds of single, identically grown Ws-2 wild type, tt12 mutant or T2 seeds of individual Pro_{BAN5}*::*$TT12_{wt}$/*tt12* and Pro_{BAN5}*::*$TT12_{E290A}$/*tt12* transformants were incubated for 3d with 2% (w/v) DMACA in 3M HCl/50% (w/v) MeOH and washed in excess 70% EtOH. The dark color of Ws-2 and $TT12_{WT}$/*tt12* seeds indicates the presence of PAs while $TT12_{E290A}$/**tt12** like the original tt12 seeds remain brown suggesting absence of PAs. (C) Histochemical vanillin staining of immature seeds in the torpedo stage of embryo development. Again, T2 seeds of *tt12* mutants transformed with Pro_{BAN5}*::*$TT12_{E290A}$ do not produce PAs in contrast to lines complemented with the wild-type TT12 version. Bar = 100 µm.

Figure 4. The E290A mutation of TT12 does not affect tonoplast localisation. The subcellular localisation of the TT12 variants was inspected in root hairs (A) or root tip cells (B, C) of T1 plants (Ws-2) transformed with *Pro$_{dual35S}$:TT12$_{WT}$-GFP*(TT12$_{wt}$-GFP) or *Pro$_{35S}$:TT12$_{E290A}$-YFP* (TT12$_{E290A}$-YFP) by CLSM. GFP and YFP images (averages of 16 frames) were both captured using the 488 nm Ar laser, 500-520 nm fluorescence emission (DD488/543 beam splitter) and were subsequently false-colored in green. In (A, B) the corresponding DIC images are presented. In (C) cell walls of root cells were counterstained with 50 µM propidium iodide whose fluorescence emission was detected between 650-720 nm and which was false-colored in red. Merged images of the green and red channels are depicted. Irrespective of the presence or absence of the E290 mutation, TT12-FP fluorescence is seen on membranes that correspond to the tonoplast as judged by the characteristic presence of 'cavities' (asteriks) which delineate cytosolic material or the nucleus, bulbs (triangles) (Reisen *et al*, 2005), and transvacuolar strands (arrows). Bars = 10 µm.

Figure 5. Flavonoid composition of mature seeds. Flavonol and proanthocyanidin content of seeds from *tt12* mutant, wild-type, pBAN::TT12$_{wt}$/*tt12* and pBAN::TT12$_{E290A}$/*tt12*. Values represent the average and SE of three independent measurements: G, glucoside; H, hexoside; I, isorhamnetin; K, kaempferol; Q, quercetin; R, rhamnoside; sol, soluble. Flavonols were extracted with acetone:MeOH:water:TFA (40:32:28:0.05; v:v) and measured by LC-MS and proanthocyanidin after acid catalyzed hydrolysis (Routaboul *et al*, 2006) (***:$p<0.001$).

Figure 6. Cyanidin-3-glucoside reduces H^+-pumping in yeast vesicles expressing *TT12*. Depicted is fluorescence quenching of the H^+-sensitive dye ACMA indicating ATP-dependent acidification of vesicles isolated from empty vector (A), *TT12$_{WT}$* (B), and *TT12$_{E290A}$* (C) transformed yeasts. Addition of ATP and NH_4Cl to initiate or reverse H^+ pumping are marked by asterisks and arrows, respectively. Quenching curves were recorded in the presence (circles) and absence of MgATP (squares) and without or with cyanidin-3-glucoside (open and filled symbols, respectively) added to the assay.

Supplementary Figure 1. WebLogo (weblogo.berkley.edu) plots of the boxes 1-5 containing amino acids conserved in all Arabidopsis MATE proteins as highlighted in Figure 1 (by red bars). The amino acids chosen for mutagenesis are highlighted by asterisk and the position number within TT12 is given.

Supplementary Figure 2. Histochemical vanilin staining of immature seeds of all SDM TT12 mutants in the torpedo stage of embryo development. T2 seeds of tt12 mutants transformed with *Pro$_{BAN5}$::TT12$_{mut}$*. Bar = 100 µm.

Supplementary Figure 3. **Subcellular localization of YFP-TT12$_{mut}$ fusion proteins, transiently expressed in *Nicotiana benthamiana*.** Protoplasts or epidermal cell strips removed from the leaf

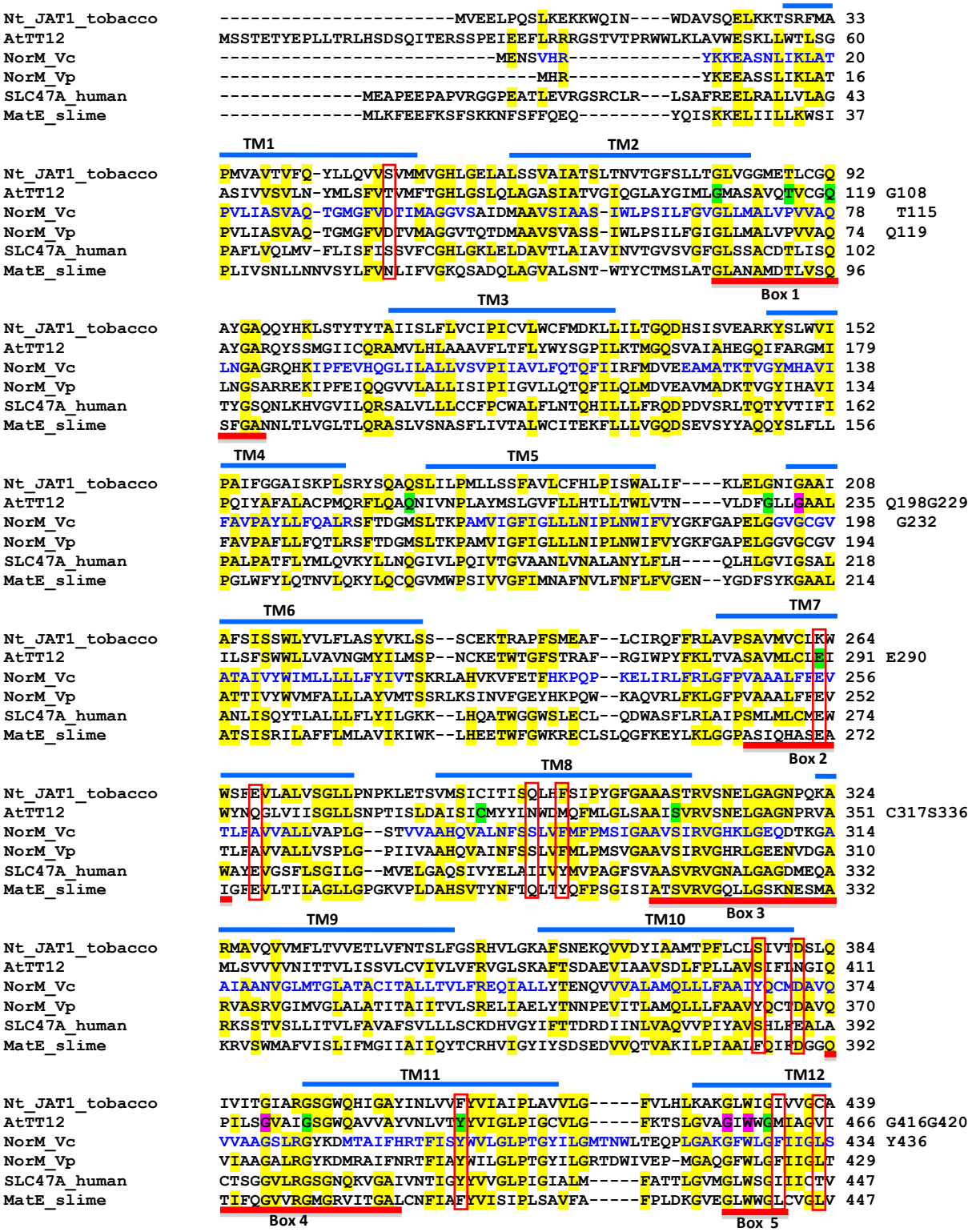
surface were inspected by epifluorescence (T115A) or by CLSM. Depicted are superimposed images exhibiting YFP and chlorophyll fluorescence false-colored in green and reed, respectively, together with the corresponding differential interference contrast images. The uppermost panel shows the $TT12_{WT}$ controls exhibiting tonoplast localisation fort he protoplast or the epidermal cell system.

Bars = 10 µm $TT12_{WT}$ (protoplast), 50 µm $TT12_{WT}$ (epidermis),10 µm $TT12_{G108I}$, 50 µm $TT12_{T115A}$, 10 µm $TT12_{Q198A,\ E290A,\ C317A,\ G229I,\ G420I}$, 50 µm $TT12_{S336A,\ Q119A,\ Y436A}$, $_{G229A,\ G229Q,\ G232Q,\ G420A,\ G420Q}$,$_{G456I,\ G456Q}$

Supplementary Figure 4. Localisation and expression of *TT12* and $TT12_{E290A}$ in yeast cells. (A) Wild-type *TT12:GFP5* and the $TT12_{E290A}$*:GFP5* variant were expressed in *S. cerevisiae* YPH499 from a 2μ plasmid under the control of the constitutive pma1 promoter. Transformed yeast were grown in selective medium to the exponential phase and incubated for 15 min with 8 µM of the endocytotic tracer FM4-64. Depicted are confocal laser scanning microscope images of yeast expressing *TT12:GFP5* (upper panel) and $TT12_{E290A}$*:GFP5* (lower panel). Single optical sections were obtained using a TCS SP2-x1 full spectrum confocal microscope with a 63x oil lens. GFP and FM4-64 were excited with a 488 nm Ar laser and fluorescence emission images averaged over eight frames were captured in independent channels (500-520 nm and 620-750 nm for GFP and FM4-64, respectively; DD488/543 beam splitter). GFP and FM-64 fluorescence images were false-colored in green and red, respectively. 'merge' represents the overlay of the green and red channels. DIC, differential interference contrast image of the depicted yeast cell. Bar = 2 µm.

Supplementary Table 1. Oligonucleotides used for site-directed mutagenesis. fwd – forward primer, rev – reverse primer.

Figure 1.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NorM_Vc
NorM_Vp
SLC47A_human
MatE_slime
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
TM9
TM10
TM11
TM12
Box 1
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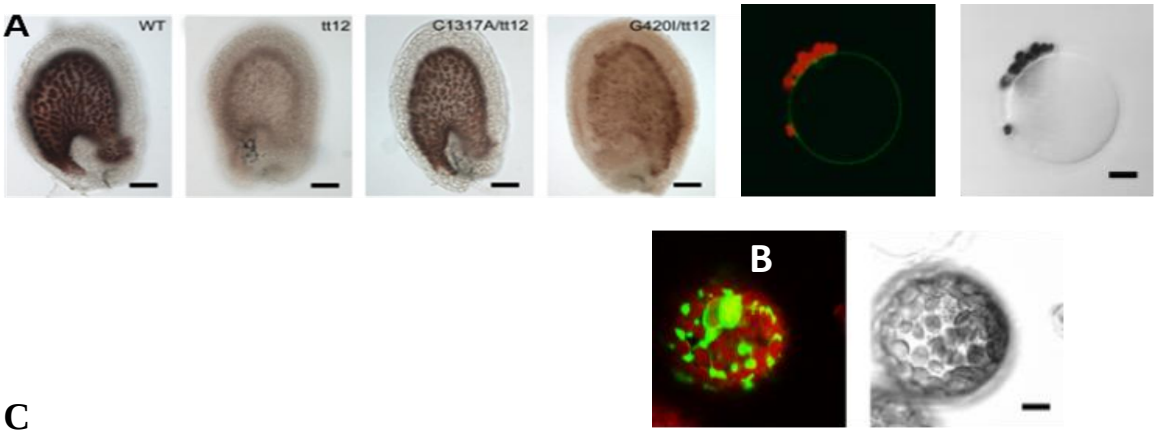
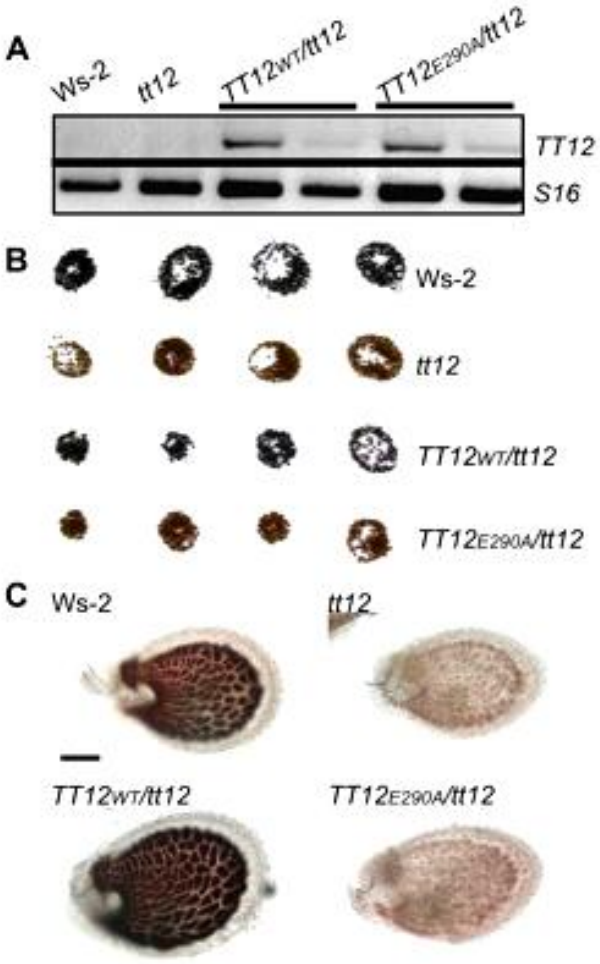
Figure 2.



C

Mutation	***tt12* complementation**	**vacuolar targeting**	**Interpretation**
Q119A, Q198A, C317A, S336A, Y436A	Similar to Ws wild-type	Yes, similar $TT12_{WT}$	Conserved residues neither critical for function nor for targeting
T115A	partial	Yes, similar $TT12_{WT}$	Residue partially affecting transport function
E290A	none	Yes, similar $TT12_{WT}$	Residue critical & limiting transport function
G108I	none	No, other endomembranes	Replacement G with I alters TT12 targeting
G229I	none	Similar to $TT12_{WT}$ and on other endomembranes	Polar residue required for proper targeting.
G229A, G229Q	partial, patchy staining	No, other endomembranes	
G232I	none (dark, greenish)	No, other endomembranes	Replacement G with I alters TT12 targeting
G232Q	Similar to Ws wild-type	Yes, similar $TT12_{WT}$	Polar residue required for proper targeting.
G416I, G416Q	none, weak	n.l.	
G420I	none	No, other endomembranes	Polar residue required for proper targeting.
G420A, G420Q	partial, patchy staining	Similar to $TT12_{WT}$ and on other endomembranes	
G456I, G456Q	partial, patchy	Similar to $TT12_{WT}$ and on other endomembranes	Polar residue required for proper targeting.
W458A	Similar to Ws wild-type	n.d.	Conserved residues criticalk for function

Figure 3.


A
Ws-2
tt12
TT12WT/tt12
TT12E290A/tt12
TT12
S16
B
Ws-2
tt12
TT12WT/tt12
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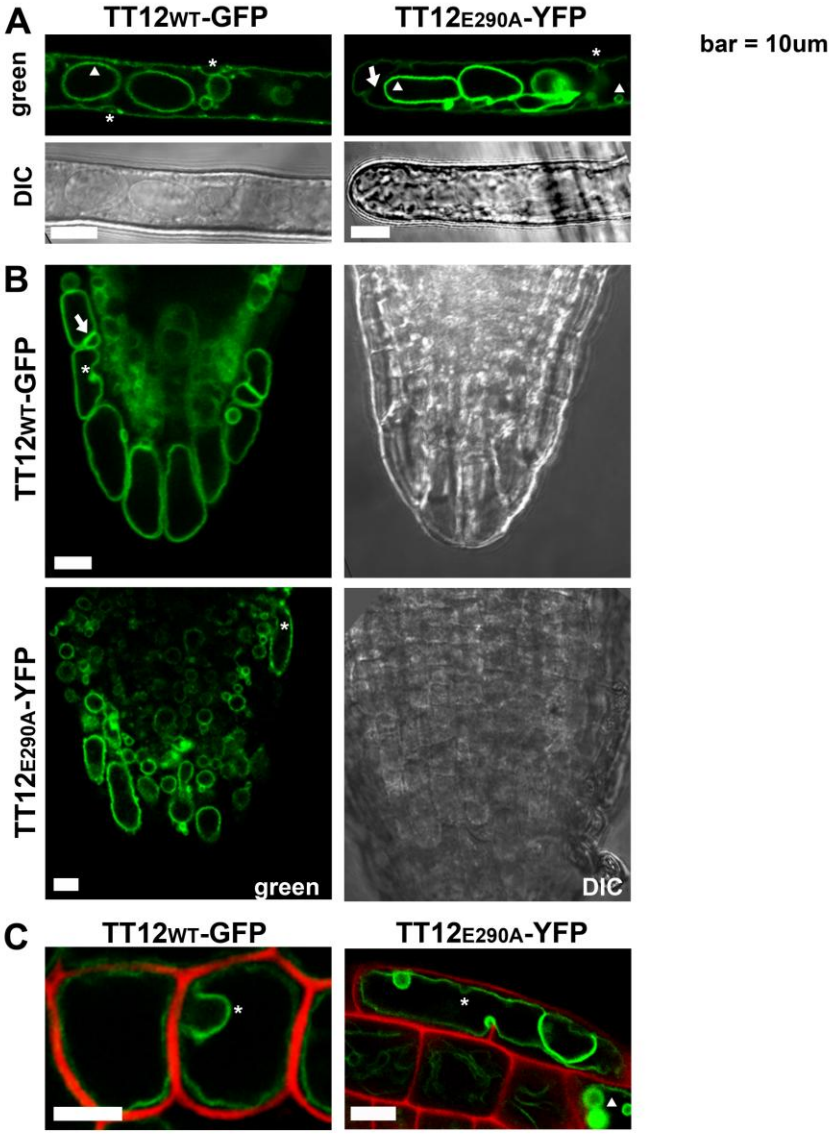
C
Ws-2
tt12
TT12WT/tt12
TT12E290A/tt12

Figure 4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TT12WT-GFP
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bar = 10um
green
DIC
B
TT12WT-GFP
TT12E290A-YFP
green
DIC
C
TT12WT-GFP
TT12E290A-YFP

Figure 5.

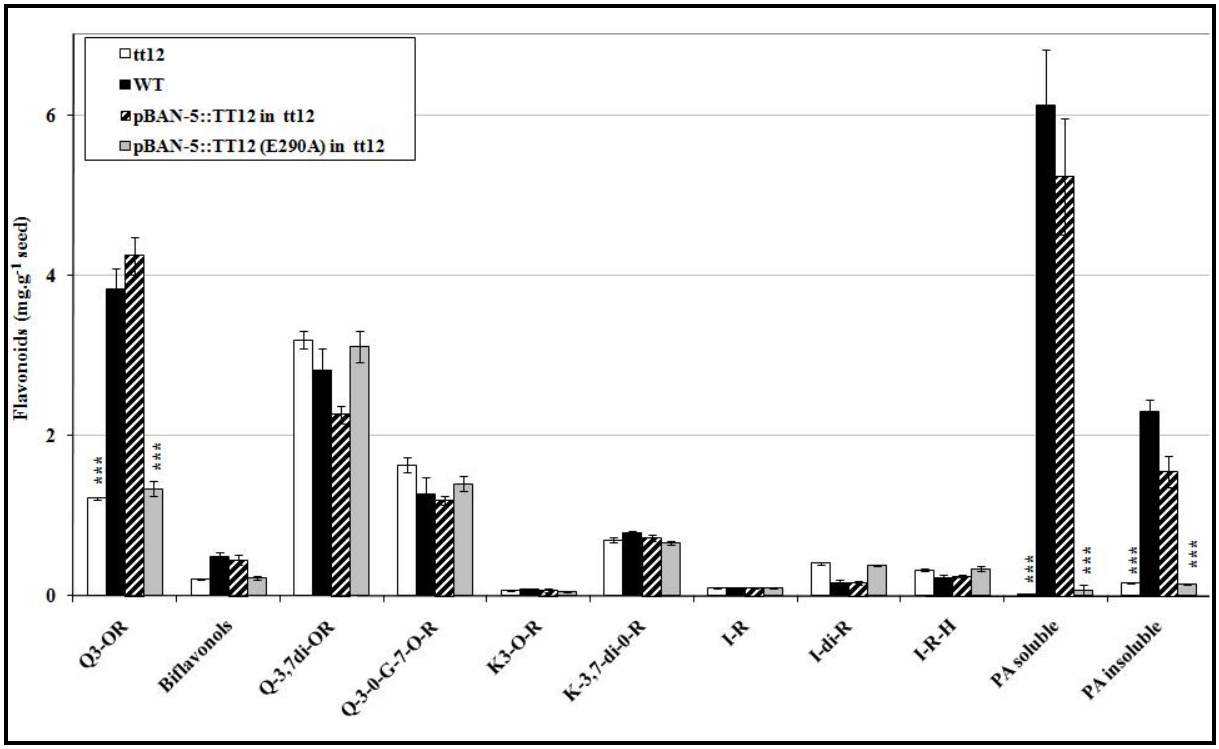
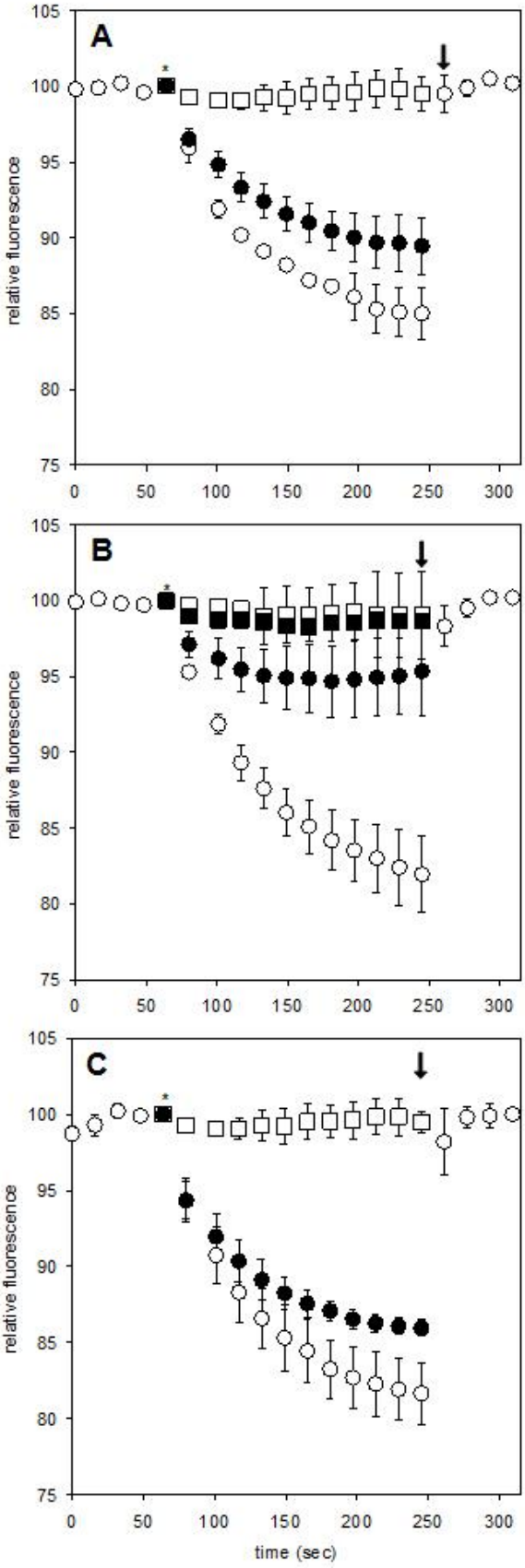
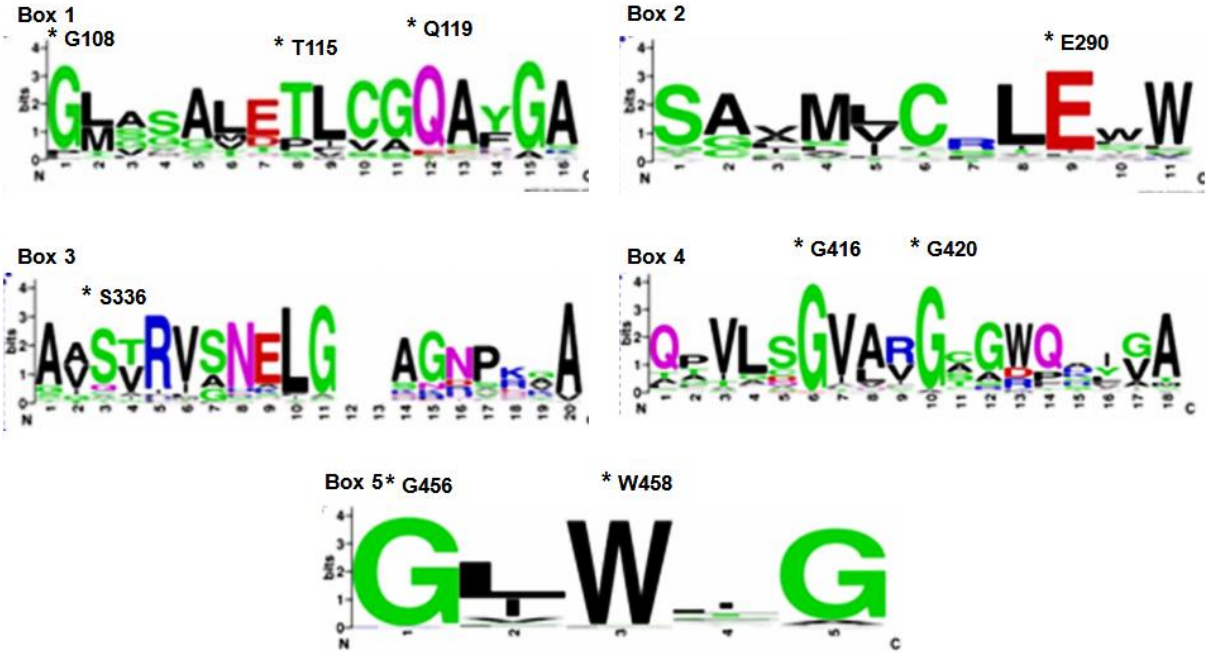

tt12
WT
pBAN-5::TT12 in tt12
pBAN-5::TT12 (E290A) in tt12
Flavonoids (mg.g-1 seed)
0
2
4
6
***
***
***
***
Q3-OR
Biflavonols
Q-3,7di-OR
Q-3-0-G-7-O-R
K3-O-R
K-3,7-di-0-R
I-R
I-di-R
I-R-H
PA soluble
PA insoluble

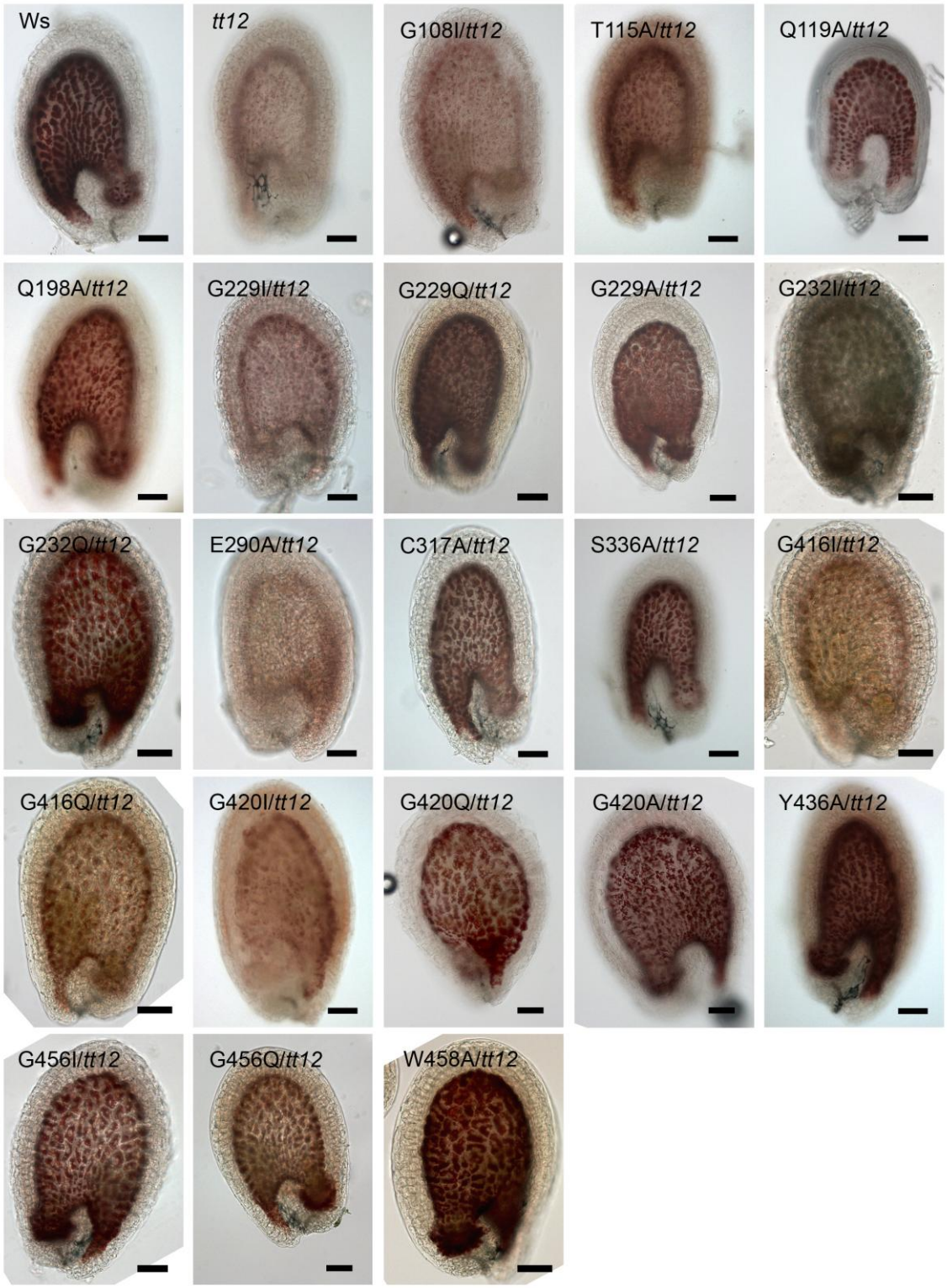
Figure 6.


A
B
C
relative fluorescence
time (sec)
105
100
95
90
85
80
75
0
50
100
150
200
250
300

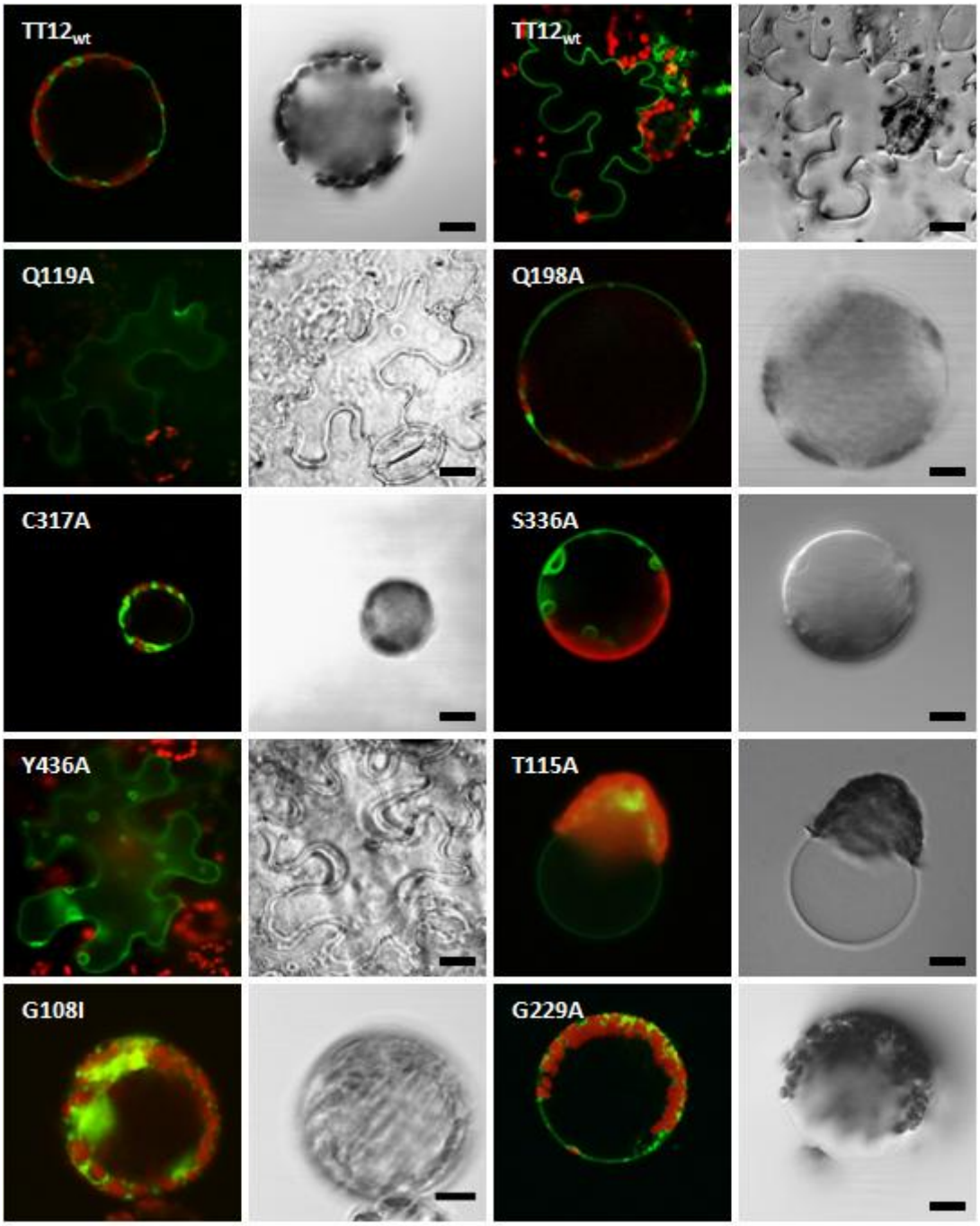
Supplementary Figure 1.

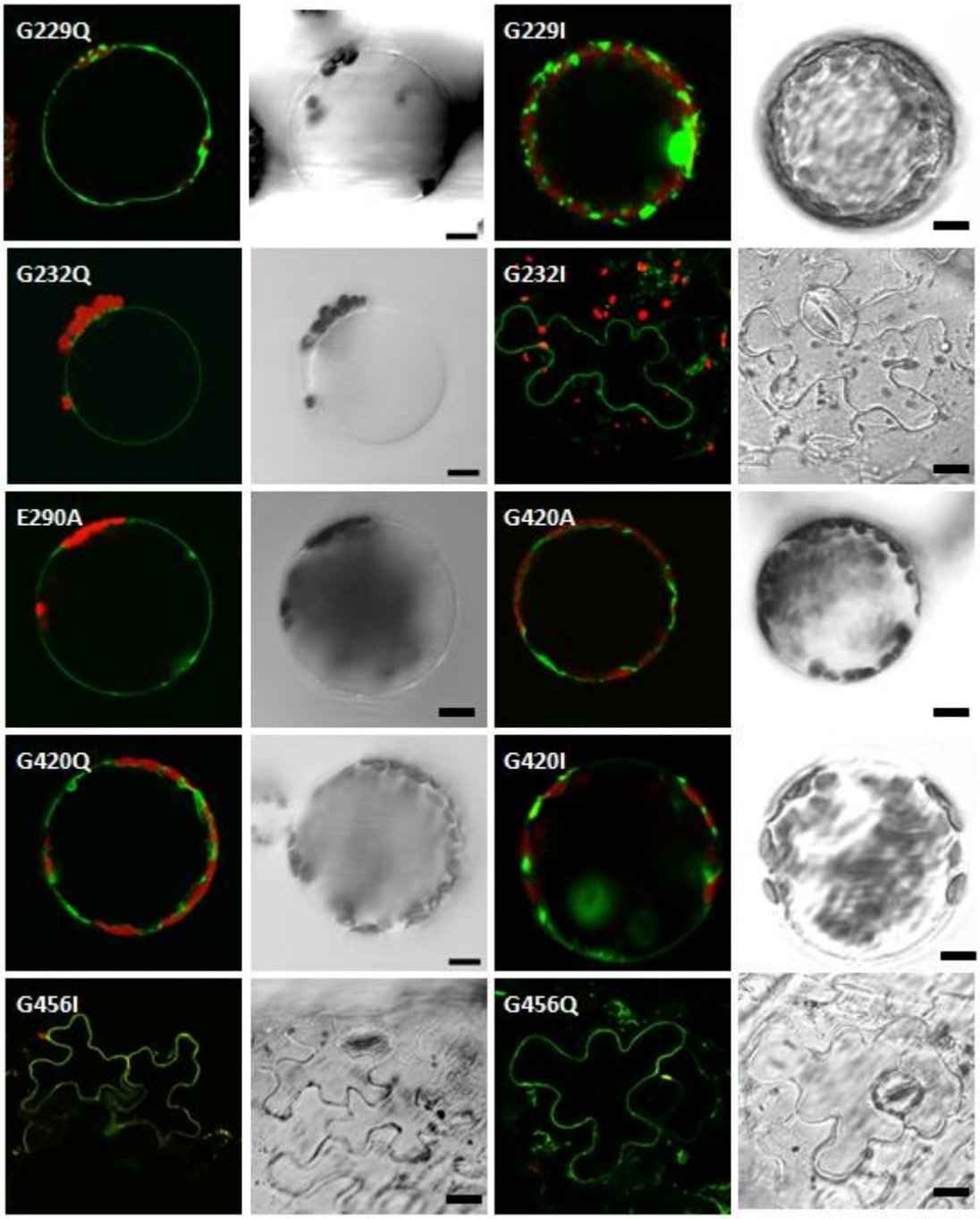


Supplementary Figure 2.

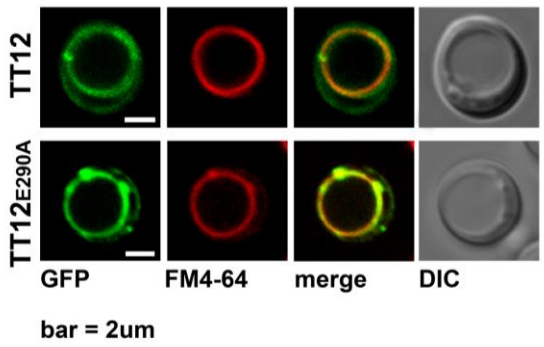


Supplementary Figure 3.




G229Q
G229I
G232Q
G232I
E290A
G420A
G420Q
G420I
G456I
G456Q

Supplementary Figure 4.



Supplementary Table 1.

Name of the primer	Number of oligonucleotides	Primer sequence
G108I fwd	29	CGGTATCATGTTAATAATGGCGAGCGCGG
G108I rev	29	CCGCGCTCGCCATTATTAACATGATACCG
T115A fwd	29	GCGCGGTCCAAGCAGTGTGTGGTCAAGCG
T115A rev	29	CGCTTGACCACACACTGCTTGGACCGCGC
Q119A fwd	35	CGGTCCAAACAGTGTGTGGTGCGGCGTACGGAGCG
Q119A rev	35	CGCTCCGTACGCCGCACCACACACTGTTTGGACCG
Q198A fwd	31	GGTTTCTTCAGGCTGCGAACATAGTGAACCC
Q198A rev	31	GGGTTCACTATGTTCGCAGCCTGAAGAAACC
G229I fwd	26	CGTGCTGGATTTCATCTTGCTTGGGG
G229I rev	26	CCCCAAGCAAGATGAAATCCAGCCACG
C317A fwd	41	CGCTATTTCGATTGCCATGTATTACTTGAATTGGGATATGC
C317A rev	41	GCATATCCCAATTCAAGTAATACATGGCAATCGAAATAGCG
S336A fwd	40	GGTCTAAGTGCAGCAATCGCTGTGCGAGTGAGCAATGAGC
S336A rev	40	GCTCATTGCTCACTCGCACAGCGATTGCTGCACTTAGACC
G420I fwd	34	GGGGTTGCTATTATCAGTGGGTGGCAAGCAGTGG
G420I rev	34	CCACTGCTTGCCACCCACTGATAATAGCAACCCC
Y436A fwd	42	GGCTTATGTGAATCTTGTTACGTACGCTGTCATTGGTCTTCC
Y436A rev	42	GGAAGACCAATGACAGCGTACGTAACAAGATTCACATAAGCC
G229Q fwd	26	CGTGCTGGATTTCCAGTTGCTTGGGG
G229Q rev	26	CCCCAAGCAACTGGAAATCCAGCACG
G229A fwd	26	CGTGCTGGATTTCGCCTTGCTTGGGG
G229A rev	26	CCCCAAGCAAGGCGAAATCCAGCACG
G420Q fwd	34	GGGGTTGCTATTCAGAGTGGGTGGCAAGCAGTGG
G420Q rev	34	CCACTGCTTGCCACCCACTCTGAATAGCAACCCC
G420A fwd	34	GGGGTTGCTATTGCGAGTGGGTGGCAAGCAGTGG
G420A rev	34	CCACTGCTTGCCACCCACTCGCAATAGCAACCCC
G232I fwd	34	GGATTTCGGCTTGCTTATCGCGGCTCTGATTCTC
G232I rev	34	GAGAATCAGAGCCGCGATAAGCAAGCCGAAATCC
G232Q fwd	34	GGATTTCGGCTTGCTTCAGGCGGCTCTGATTCTC
G232Q rev	34	GAGAATCAGAGCCGCCTGAAGCAAGCCGAAATCC

CHAPTER IV: FUNCTIONAL ANALYSIS OF ARABIDOPSIS MATE PROTEINS

4. 1. INTRODUCTION

In chapter II we studied structure and function of conserved amino acid residues of TT12, a MATE transporter involved in the vacuolar anthocyanin/H^+-antiport mechanism active in proanthocyanidin-accumulating cells of seed coat endothelium. In this chapter we present attempts to identify novel flavonoid transporters from the Arabidopsis MATE gene family.

One of the problems connected with redundant gene families such as MATE transporters in Arabidopsis is that several genes may exert the same function. As a result, single gene knock-outs do not necessarily display any change of phenotype. We hypothesized that MATE transporters linked to TT12 might be vacuolar anthocyanin or flavonol transporters in Arabidopsis. Since the vacuolar flavonoid/H^+ antiport mechanism of TT12 was characterised we expected that MATE transporters phylogenetically related to *TT12* could encode for putative flavonoid transporters. *TT12* is specifically expressed in the seed coat in cells actively involved in PA biosynthesis. *TT12* expression is thus identical to the expression pattern of BANYLUS. Since absence of *TT12* affects the *banylus* anthocyanin phenotype in double mutant seeds and both *tt12* and *tt12ban* seed phenotypes are easily scorable we planned to use this strategy to verify potential anthocyanin transporters.

Figure 4.1. Phylogenetic analysis of MATE transporters (http://www.genebee.msu.su). The AGI codes or Genebank entries are as follows: AtDTX35, At4g25640; SlMTP77, AAQ55183.1; AtDTX28, At5g44050; AtDTX31, At1g12950; AtTT12, At3g59030; At, *A. thaliana*; Sl, *Solanum lycopersicum*.

We selected three candidate genes based on their phylogentic relationship to TT12 and if they or their homologues had been previously described to be specifically induced or expressed in the literature. These were as follows:

AtDTX35 (At4g25640) has been identified as the closest homologue of a tomato MATE which is transcriptionally upregulated in the *ANT1*tomato mutant (Fig. 4.1). *ANT1* encoding proteins

involved in both early and later steps of anthocyanin biosynthesis was initially identified in screens of activation-tagged lines and showed intense purple pigmentation from the early stages of growth, indicative of anthocyanin hyperaccumulation. Overexpression of *ANT1* in tomato triggered the upregulation of genes encoding proteins involved in anthocyanidin biosynthesis, glycosylation and transport of anthocyanins into the vacuole (Mathews *et al*, 2003).

AtDTX31 (At1g12950) is transcriptionally upregulated in Arabidopsis roots, under salt stress conditions (Arabidopsis eFP browser). Cytosolic accumulation of Na^+ is harmful to many biological processes so the intracellular and intercellular transport of this ion needs to be controlled. AtDTX31 has been shown to be 10 fold upregulated in response to salt stress and showed similarity to proteins from tomato which are predominantly expressed in fruit tissues and play roles in diverse biological functions e.g. fruit ripening (Maathuis, 2006).

AtDTX28 (At5g44050) has been shown to be induced after UV-B treatment in wild-type Arabidopsis plants but not in (i) *cop1* mutants *(constitutively photomorphogenic 1)*, which is a negative regulator of photomorphogenesis in Arabidopsis functioning as an E3 ubiquitin ligase and (ii) *hy5mutants (elongated hypocotyls 5)*, a key regulator of photomorphogenesis required for tolerance to UV-B (Oravecz *et al*, 2006). UV-B is known to inhibit growth of the hypocotyls, change specific gene expression and induce flavonoid production (Frohnmeyer and Staiger, 2003, Ulm and Nagy, 2005). Furthermore, UV-B dependent, *cop1* and *hy5* blocked induction of DTX28 expression correlates with increased transcript abundance of genes involved in biosynthesis of flavonol and *AtMYB12* which is a transcription factor activating flavonol biosynthesis (Mehrtens *et al*, 2005). Consequently, we hypothesized that At5g44050 encodes a vacuolar flavonol transporter.

Here, we demonstrate our molecular and biochemical analyses of these three putative flavonoid transporters: (i) AtDTX35 was assumed to be involved in anthocyanin transport, (ii) AtDTX31 presented an *in silico* gene expression pattern that hinted at a link to salt stress and (iii) AtDTX28 showing high homology to proteins expressed in flavonol biosynthesis.

4.2. MATERIAL AND METHODS

4.2.1. PLANT MATERIAL AND GROWTH CONDITIONS

Unless otherwise stated, all soil (Einheitserde, type ED73, Gebr. Patzer GmbH & Co. KG, Schopfheim, Germany) grown plants were planted one to a pot and grown in a controlled environment chamber under short day conditions: 8h, 120 μmol photons m^{-2} s^{-1}, 22°C, 60% relative humidity.

In vitro grown plants were grown on MS supplemented with 1% sucrose in a chamber with 16h of light and 22ºC.

Hydropnically grown plants were first cultivated for 2 weeks on MS enriched with 3% sucrose in *in vitro* conditions as described above and then transferred to hydroponics (Araponics, http://www.araponics.com/) and grown in tap water for 2-3 weeks till the roots are 2-3 cm long in a controlled environment chamber as described above. After that time, the tap-water was exchanged with hydroponics medium: 4 mM KNO_3, 1.2 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$, 0.8 mM KH_2PO_4, 0.8mM NH_4Cl, 0.048 mM Fe(III)EDTA, 75 µM H_3BO_3, 25 µM $MnSO_4*H_2O$, 2.5 µM $CuSO_4*5H_2O$, 2.5 µM $ZnSO_4*7H_2O$, 0.75 µM $(NH_4)_6Mo_7O_{24}*4H_2O$, 125 µM KCl (modified from Massonneau *et al*, 2001) and the plants were grown for another 2 weeks before being used for experiments (chapter 4.2.6.8.1.2).

Nicotiana benthamiana plants were propagated from seeds under greenhouse conditions. After 7 days, plants were transplanted, one to a pot, and grown for another 2-3 weeks until they were experiment ready (4.2.5.1).

Unless otherwise stated, all the experiments were conducted on Arabidopsis Col-0 plants.

Seeds of all plants were surface sterilized for 4-6 h in a chamber containing vapours of sodium hypochlorite and HCl and then stratified for 2 days in 4ºC.

The DTX35 mutant in the Col-0 background was obtained from the SALK collection (lines SALK_1199966, SALK_095202, SALK_122274) T-DNA insertion mutant collection (Alonso *et al*, 2003). The mutants carry T-DNA insertions in the first exon (SALK_095202) and last intron (SALK_122274, SALK_1199966) of the gene. Homozygous mutants were identified by using a triplex primer PCR. The wild-type allele (~ 0.9 kB) was amplified with the primers for SALK_1199966 fwd: 5' CGTCCACGAGCAGCTAAGTAC, rev: 5' TGTCA GAAGTGGGGTTTGAAC; for SALK_095202 fwd: TCTACCAGACCTTCACAACCG, rev: 5' CAAGCTGCAAGGATTCAAAAC and for SALK_122274 fwd: 5' GAGCAATTC AAACCAGCTGAG, rev: 5' GCGCTTCAAACGTTGTTACTG. The mutant allele (~ 0.5 kB) was identified by using Lbb1.3 T-DNA specific primer and SALK_1199966rev, SALK_095202rev, SALK_122274rev, respectively.

The DTX31 mutant in the Col-0 background was obtained from the SALK collection (SALK_103884) (Alonso *et al*, 2003). The mutant carries a T-DNA insertion in the DTX31 gene in the third intron. Homozygous mutants were identified by triple primer PCR using DTX31_fwd (5'ACATACGCTACAACTGCTTGC) and DTX31_rev (5' TAATTGTACGTGTGGAGAGGC) and Lbb1.3 T-DNA specific primers.

The DTX28 mutant in the Col-0 background was obtained from the Gabi-Kat collection (line GK-094E09). The mutant carries a T-DNA insert in the sixth intron. Homozygous mutants were isolated by PCR using five different type of primers (dtx28_fwd 5'ACGAGT GGCAAATGAATTAGGAGCAGG; dtx28_rev 5' ACCATGGTTCCTCCGAAGATCATACCC) and GABI KAT T-DNA specific primers (GABI_ 2588 5' CGCCAGGGTTTTCCCAGTCACGAC;

GABI_ 3144 5' GTGGATTGATGTGATATCTCC; GABI_ 8760 5' GGGCTACAC TGAATTGGTAGCTC).

4.2.2. NUCLEIC ACID ANALYSES

4.2.2.1. GENOMIC DNA ISOLATION

For genomic DNA isolation the simple and rapid method was used adapted from Edwards *et al*, (1991). In brief, the leaf tissue was ground in an eppendorf tube with a plastic pestle for 20 sec and 400 µl of buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the grinding was continued until the tissue was well macerated. After that the tubes were vortexed for 5 sec and left at RT until all samples were ready. They were then placed into the thermoblock for 10 min at 50ºC with vigorous shaking and centrifuged (5 min, 14000 rpm, RT). 300 µl of supernatant was transferred to a fresh tube and 300 µl of isopropanol was added, mixed and left at RT for 2-10 minutes. Subsequently, samples were placed at -20ºC for 20-30 min, then centrifuged (15 min, 14000 rpm, RT) and supernatant was removed. Next the pellet was rinsed with 70% EtOH, drained and air-dried. Consequently, the pellet was resuspended in 100 µl of TE and 1 µl was used for further analyses.

4.2.2.2. TOTAL RNA EXTRACTION

RNA was extracted from leaves and roots of seedlings (14 days-old) grown on half-strength MS enriched with 1% sucrose and solidified with 0.9% phytoagar . Total RNA was isolated from plants using either the RNeasy Plant Mini Kit (Qiagen) or SV Total RNA Isolation System (Promega AG, Dübendorf, Switzerland) according to manufacturer's protocol.

4.2.2.3. cDNA SYNTHESIS AND GENE EXPRESSION ANALYSES

First strand cDNA was synthesized using M-MLV (H^-) reverse transcriptase (Promega) as specified by the manufacturer. The semi-quantitative RT-PCRs were performed in a final volume of 50 µL (1 µl cDNA, 1.25 U GoTaq DNA polymerase (Promega), 1 x PCR buffer, 0.2 mM of each dNTP, 0.2 µmol of each primer. The number of sqPCR cycles was determined for the linear range of the constitutively expressed *RPS16A* gene (encoding a 40S ribosomal protein). The *RPS16A* primer pair (*RPS16A*$_{fwd}$ 5' GGCGACTCAACCAGCTACTGA and *RPS16A*$_{rev}$ 5'CGGTAACTCTTCTGGT AACGA) amplified a 0.8 kB fragment of the cDNA.

The *AtDTX35* (At4g25640) primer pair (*AtDTX35*_fwd 5'ATGGATCCGACGGCGCCGTTGCTT ACAC and *AtDTX35*_rev 5' CGCAAGTATATCCTTGGATGTCGTTCA) amplified a 1.4 kb fragment of the cDNA. The *AtDTX31* (At1g12950) primer pair (*AtDTX31_fwd* 5' ATGGAGAAAGATAATGACTTCAAGGATC, *AtDTX31_rev* 5' GTTCAAAAGAGTCTCTTTCT CTGCAGGT) amplified a 1.5 kb fragment of cDNA. The *AtDTX28* (At5g44050) primer pair (*AtDTX28_fwd* 5' ATGGGAGAGAGAGACGACGAAGCAGAAGG, *AtDTX28_rev* 5' CTTTCTTGCGTCTGATACAGACCATTTG) amplified the open reading frame (~1.5 kb) of the gene.

4.2.3. PLASMID CONSTRUCTIONS

4.2.3.1. DTX35

The *DTX35* gene (1463 bp without stop codon) was amplified using DTX35_fwd (5' ATGGATCCGACGGCGCCGTTGCTTACAC) and DTX35_rev (5' CGCAAGTATATCCTT GGATGTCGTTCA) primers by RT-PCR and cloned into pCR8 (pCR®8/GW/TOPO® TA Cloning Kit), following the manufacturers guidelines. Subsequently, the DTX35 cDNA was sub-cloned into pMDC83 (GFP vector), pMDC83Ban (Curtis and Grossniklaus, 2003) and pE101 (YFP vector) (Earley *et al*, 2006) using an LR clonase reaction (Invitrogen®), following the manufacturers guidelines.

In order to analyse promoter activity, a β-glucoronidase fusion was created. A 1.9 kB fragment immediately upstream of the start codon of DTX35 was amplified by PCR of genomic DNA (Col-0), using primers DTX35prom_fwd (5' TAGATGATTCATTTACTGAAGAAATGGC) and DTX35prom_rev (5' CTTCTGAAAATGATTAACCAAAACGC). The amplified promoter fragment was cloned into the pCR8 vector and sub-cloned into pMDC163 (Curtis and Grossniklaus, 2003) using an LR clonase reaction.

To construct the 2µ yeast expression vector pNEV-Ura (Sauer and Stolz, 1994) containing the *DTX35* cDNA we performed recombination-mediated PCR-directed plasmid construction *in vivo* in yeast (Oldenburg *et al*, 1997). DTX35 cDNA was amplified with primers containing 14 nucleotides homologous to each end of the linearized pNEV-Ura and containing *att* sites (primers Rec-entrpma_up 5' TTATACACACATTCAAAAGAAAGAAAAAAAATATACCCCAGCCGCGGCC GCGTACAAAAAGCAG and Rec_entrpma_low 5' TTAAGGTGTGTGTGTGGATAAAATATT AGAATGACAATTCCCGGCGCGCCGTACAAGAAAGCTGG, the regions of homology to pNEV-Ura underlined), gel-purified and co-transformed into the yeast YPH499 strain in a vector: insert ratio of 1:6 (Gietz and Woods, 2002). Transformants were selected on minimal synthetic dropout medium

lacking uracil (SD-Ura). The presence of the insert was verified by yeast colony PCR (Ling *et al*, 1995). Briefly, yeast colony was touched by sterile pipette tip and rinsed with 10 µl zymolase solution (2.5 mg/ml zymolase (Seikagaku, Kogyo), 1.2 M sorbitol, 0.1 M sodium phosphate, pH 7.4) by pipetting up and down 3-5 times and incubated for 5 min at 37ºC. The 2 µl spheroplasted yeast cells was used for 50 µl PCR reaction (94ºC 3min, 30 cycles of 94 ºC 30 sec, 58 ºC 30 sec, 72 ºC 1.5 min, 72 ºC 7 min).

RNAi silencing contruct was obtained using pKANNIBAL hairpin vector applied widely for producing of intron-spliced hairpin RNAs (Wesley *et al*, 2001). pKANNIBAL contains a 35S-driven inverted repeat cassette with restriction sites for directional cloning of fragments from the target gene. The gene of interest (248 nt fragment of *DTX35*) was PCR-amplified using primers containing *BamHI* and *XhoI* restriction sites (DTX35_958fwd 5' CCTCCAACAGCCACACCAGAAACAACCG and DTX35_1205rev 5' GTATCCAATGAGCTTGGCCTAGGCCGT) matching those in the hairpin vector and cloned into the hairpin cassette. This cassette was then restricted with *NotI* and cloned into pGreen 0179 binary vector (Hellens *et al*, 2000). This construct was introduced into the *Agrobacterium tumefaciens* GV3101 strain by electroporation, using a Genepulser (2.5 kV; 100Ω; 25 µF, Bio-Rad, Reinach, Switzerland)**.** Co-transformation of Col-0 and PAP1-D with pSoup vector was performed as previously described (Nesi *et al*, 2000). pGreen can replicate in Agrobacterium only if pSoup is co-resident in the same strain. pSoup provides replication functions in trans for pGreen (Hellens *et al*, 2000).

4.2.3.2. DTX31

To clone the Arabidopsis *DTX31* cDNA (1566 bp without stop codon) we performed RT-PCR using DTX31_fwd (5' ATGGAGAAAGATAATGACTTCAAGGATC) and DTX31_rev (5' GTTCAAAAGAGTCTCTTTCTCTGCAGGT) primers. The amplified sequence was cloned into pCR8 (pCR®8/GW/TOPO® TA Cloning Kit) and sub-cloned into pMDC83 vector (GFP vector) (Curtis and Grossniklaus, 2003) using an LR clonase reation (Invitrogen®) according to the manufacturer's protocol.

To analyse promoter activity, a β-glucoronidase fusion was generated. A 1.2 kB fragment upstream of the start codon of DTX31 was amplified by PCR on genomic DNA (Col-0), using DTX31prom_fwd (5' TATCTTTTGTTGATCGTGATAATAATG) and DTX31prom_rev (5' TCTCCGACGACAAGAGAAAGAG) primers. The amplified promoter fragment was inserted into pCR8 vector and sub-cloned into pMDC163 vector (Curtis and Grossniklaus, 2003) using an LR clonase reaction.

4.2.3.3. DTX28

To clone the *DTX28* gene of Arabidopsis we performed an RT-PCR with DTX28_fwd (5' ATGGGAGAGAGAGACGACGAAGCAGAAGG) and DTX28_rev (5' CTTTCTTGCGTCTGATA CAGACCATTTG) primers and cloned the amplified gene into the pCR8 vector (Invitrogen®) following the manufacturer's instructions. Subsequently, the DTX28 cDNA was sub-cloned into pMDC83 (GFP vector) (Curtis and Grossniklaus, 2003) and pE101 (YFP vector) (Earley *et al*, 2005) using LR clonase reaction (Invitrogen®).

The promoter fragment of DTX28 (790 bp) (received from R. Stracke, Bielefeld) was cloned from genomic DNA (Col-0) by using DTX28prom_fwd (5' TGAATTCAAAAAAGA AGAGAAGGTATATT) and DTX28prom_rev (5' TCCCATGGCCGATCTTCAGTAAATTAAC). The amplified promoter fragment was cloned into pCR8 vector (pCR®8/GW/TOPO® TA Cloning Kit, Invitrogen) and sub-cloned into pMDC163 (Curtis and Grossniklaus, 2003) using an LR clonase reaction.

For all constructs, PCR amplifications were carried out using the Expand High Fidelity PCR System (Roche). The correct integrating and the absence of errors in all of the gene sequences was verified by sequencing.

4.2.4. STRAINS

Various constructs were transformed into the bacterial strains DH10b (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80l*acZ*ΔM15 Δ*lacX*74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G) and DH5α (F- φ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *gal- pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1), using a conventional heat shock transformation protocol.

The yeast strain YPH 499 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-1) was used for heterologous expression of the *DTX35* cDNA. This strain was used for a yeast transformation as described previously (Gietz and Woods, 2002) and used for the isolation of microsomal membrane vesicles.

4.2.5. PLANT TRANSFORMATION METHODS

4.2.5.1. TRANSIENT GENE EXPRESSION IN NICOTIANA BENTHAMIANA

Transient expression in *N. benthamiana* leaves using infiltration of *Agrobacterium tumefaciens* GV3101 transformed with the corresponding plasmids by electroporation was performed as shown previously (Voinnet *et al*, 2003 Walter *et al*, 2004). Briefly, 4-5 mL of YEB (0.5% beef

extract, 0.1% yeast extract, 0.5% bacto peptone, 0.5% sucrose, 0.49% $MgSO_4$ x $7H_20$) or LB (Luria broth; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) medium was inoculated with transformed strain of *A. tumefaciens* GV3101 and incubated with shaking at 28ºC for 18-24 h. Cultures were pelleted (15 min, 4000 rpm, RT) and resuspended in 1 ml of AS medium (9.9 mM $MgCl_2$, 0.01M MES-KOH, pH 5.6, 0.15 mM acetosyringon). Subsequently the OD_{600} was adjusted to 0.7-0.8 with AS medium and cultures transformed with RK19 silencing vector and the binary vector of interest were mixed 1:1 and incubated for 2-4 h at RT. After that mixed cultures of *Agrobacterium* were pressed in the abaxial site of the leaves and incubated for 48-72 h at RT.

4.2.5.2. STABLE TRANSFORMATION OF ARABIDOPSIS THALIANA

Stable transformation of *Arabidopsis thaliana* was performed as follows (modified from Clough and Bent, 1998). Arabidopsis plants were grown to the flowering stage in the controlled environment (3.2.1). Unless otherwise noted, Col-0 ecotype was used for the experiments. *Agrobacterium tumefaciens* GV3101 was used in all experiments for which data are shown. Bacteria were grown for 18-24 h to stationary phase in liquid culture at 28ºC in LB containing kanamycin (50 µg ml^{-1}). Cells were harvested by centrifugation for 15 min at 4ºC at 5000 x g and then resuspended in infiltration medium (5% sucrose, 0.05% Silwet L-77) to a final OD_{600} of approximately 0.8. The inoculum was added to a beaker, plants were inverted into this suspension and kept there for 30-60 sec. Dipped plants were removed from the beaker, placed horizontally in a plastic tray and covered with a tall clear-plastic cling film to maintain humidity. Plants were left in a low light location overnight, uncovered, placed in a vertical position and returned to the controlled growth environment the next day. They were grown for a further 3-5 weeks until siliques were brown and dry.

4.2.6. ANALYTICAL METHODS

4.2.6.1. PHENOTYPIC ANALYSES

To investigate the function of a gene in flavonoid biosynthesis, 10-days-old Arabidopsis seedlings grown on half strength MS agar-solidified medium, were transferred to plates of ½ MS medium that contained 0.5 mM cinnamic acid, 100 and 200 nM quercetin and cultured for 5 days.

4.2.6.2. GUS STANING PROTOCOL

Promoter activity was visualised *in vivo* by GUS staining of Arabidopsis transformed with promoter β-glucoronidase (GUS) gene constructs as previously described (Parcy *et al*, 1998). In short,

T2 seeds of plants transformed with vectors carrying uidA:35S casette were grown for 7 days on 1/2 MS enriched with 1% sucrose. Seedlings were collected and placed in cold 90% acetone on ice. When all of them were proceeded, they were placed at RT for 20 minutes. The acetone was removed and staining buffer on ice (0.2% Triton X-100, 50 mM Na_2HPO_4 pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide) was added. The remaining staining buffer was supplemented with 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexamine salt) and it substituted the buffer without X-Gluc. The samples were then infiltrated under vacuum, on ice, for 15-20 minutes. The vacuum was released slowly and sinking of samples was verified. The infiltrated samples were incubated at 37ºC overnight. Afterwards, the staining buffer was removed and samples were treated with 20%, 35% and 50% ethanol (EtOH) at RT for 30 min each. Subsequently samples were fixed with FAA (50% ethanol, 5% formaldehyde, 10% acetic acid) for 30 min at RT. After that FAA was removed, 70% EtOH added and samples were examined or stored at 4ºC.

4.2.6.3. PREPARATION OF ROOT CROSS SECTIONS FOR LIGHT MICROSCOPY.

Roots of 10-day-old plants grown on MS were used for the preparation of root cross sections. They were stained for GUS activity (3.2.6.2.), fixed for 3 min under vacuum in 4% (v/v) glutaraldehyde and incubated in RT for 4h. Subsequently, the tissue was washed thrice with ddH_2O and dehydrated using an ethanol series (70%, 30 min; 90%, 30 min; 100%, 1h). Additionally, the final step using 100% ethanol was repeated once. Embedding of the tissue was carried out using Technowit 7100 (Heraeus Kulzer, Dübendorf, Switzerland) following the manufacturer's protocol and root sections (2-5 µm) were cut using a hand operated microtome.

4.2.6.4. DMACA STAINING

Dry T2 seeds from T1 *tt12* individuals carrying the pMDC83Ban5pr-dtx35 construct were stained following published procedures (Debeaujon *et al*, 2000) using p-dimethylaminocinnamaldehyde (DMACA, 2% (w/v) in 3 M HCl/50% (w/v) methanol) for the presence of PAs (Abrahams *et al*, 2002). After 24h incubation at RT, seeds were washed three times with 70% (v/v) EtOH. Seeds pools were then examined using a binocular microscope Nikon SMZ1500 attached to a Leica DFC 320 camera. Wild-type Col-0 and *tt12* mutant seeds were used as positive and negative controls of DMACA-staining, respectively.

4.2.6.5. RAPID PROTOPLAST ISOLATION FROM INFILTRATED LEAVES OF NICOTHIANA BENTHAMIANA

Mesophyll protoplasts from infiltrated leaves of *N. benthamiana* transformed with respective plasmids were obtained as previously described (Frangne *et al*, 2002). In short, the abaxial side of the tobacco leaves was scraped with sandpaper. The leaves were then placed in 20-25 mL of cellulose mix (1% cellulose and 0.1% pectolyase in MCP; MCP: 500 mM sorbit, 1 mM $CaCl_2$, 10 mM MES-KOH, pH 5.6) in a Petri dish, abaxial side down in the mix and incubated at 30ºC for ~3h without shaking. Subsequently the cellulose mix was discarded and replaced with MCP. Protoplasts were shaken into MCP and collected in a round bottomed Falcon tube and underlayed with 5 ml 100% Percoll cushion, followed with centrifugation (1500 rpm, 5 min, no brake, RT). After that the epidermal cell layer was removed and the sedimented protoplasts were resuspended in the remaining 100% Percoll, overlayed with 10 mL 25% Percoll and 10 mL MCP. This gradient mix was then centrifuged (1500 rpm, 5 min, no brake, 4ºC), the protoplast layer was collected and microscopic observations were performed.

4.2.6.6. SUBCELLULAR LOCALIZATION AND CONFOCAL LASER SCANNING MICROSCOPY ANALYSIS

Subcellular localisation studies of DTX35-GFP, DTX31-GFP and DTX28-GFP fusions were carried out in transient assays and after stable transformation of *Arabidopsis* plants as previously described (chapter 3.2.5.2). Microscopic observations were performed at 25ºC. A Leica DM IRE2 inverted fluorescence microscope combined with TCS SP2-x1 full-spectrum confocal microscope was used to capture single optical sections. GFP was excited with 488-nm Ar and 543-nm HeNe laser. Fluorescence emission images averaged over 6 frames were captured (500 to 520 nm for GFP) and images were false-colored in green using Adobe Photoshop 9.0.

4.2.6.7. ROOT BENDING ASSAYS

To evaluate salt tolerance and possible involvement of flavonoids in root bending we performed root bending assays. Seeds of Col-0 and *dtx31* mutant were vapor-sterilized for 4-6 h in a sealed exsiccatorchamber and stratified at 4°C for 2-4 days. Afterwards they were sown on full-strength MS media enriched with 3% sucrose, pH 5.7 and 1.2% agar. Seedlings were grown for 4 to 6 days in vertical position and then transferred to salt-containing MS media (150 mM NaCl) and grown on the inverted plates for 7-10 days. Wild-type seedlings typically continue root growth and form a hook as the root reorientates and grows downward. Lack of hook formation indicates reduced growth and salt sensitivity.

4.2.6.8. HPLC STUDIES AND SAMPLE PREPARATION (EXTRACTS AND EXUDATES)

Aliquots (50 µl) were separated and analyzed by HPLC-RID (Dionex, Switzerland). Absorbance spectra were recorded with a diode array detector scanning from 280 to 330 nm. Data integration analysis was performed using the Chromeoleon software (Dionex, Switzerland). All analyses were carried out with at least two replicates.

The chromatographic settings: Nucleosil 100-5 C_{18} column (5 µm, 2 x 250 mm, Macherey-Nagel, Düren, Germany) with precolumn; flow rate 1.00 ml min^{-1}. Solvent A: Acetonitril; solvent B: 0.1% (v/v) H_3PO_4 in H_2O. Mobile phase conditions: gradient within 25 min from 10 to 25% of A and then within 10 min from 25 to 70% of A.

4.2.6.8.1. Sample preparation (HPLC-RID)

4.2.6.8.1.1. Root and leaves extracts from plants grown hydroponically and seedlings grown on plates

Separated roots and leaves of hydroponically grown plants/seedlings or entire seedlings were ground in liquid N_2 and lyophilized to determine dry weight (10 mg). Dried material was incubated overnight in the dark at 4ºC in 500 µl of 80% (v/v) methanol and subsequently macerated with a pestle followed by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatants were transferred to a fresh tube and evaporated in a Speed-Vac centrifuge (Univapo100 ECH) with the temperature limited to a maximum of 43ºC and resuspended in 100 µl of 80% methanol.

4.2.6.8.1.2. Root exudates

Plants were grown as described in chapter 3.2.1 for 6 weeks and subjected to 150 mM NaCl stress by adding the NaCl to the Arabidopsis hydroponic medium. Media containing exudates were collected after 0h, 6h, 24 h and 48h and stored at 4ºC for period of the experiment. The C_{14} columns (Sep-Pak Classic, Waters, Ireland) were conditioned with 5 ml of 100% methanol and 5 ml of distilled water. After that 500 ml of media was run through column using Ecoline pump (Ismatec, Switzerland) and the column was washed again with 10 ml of water. 2 ml of fresh 80% methanol was used to elute the substrates from the column. These 2 ml were then evaporated using Speed-Vac (Univapo100 ECH) with maximum temperature limited to 43 ºC and the remaining pellet was resuspended in 100 µl of fresh 80% methanol.

4.2.6.9. PREPARATION OF YEAST MEMBRANE VESICLES AND IN VITRO TRANSPORT STUDIES

Yeast membrane vesicles were isolated as previously described (Tommasini *et al*, 1996) with the modifications reported by Klein *et al*, 2002. Briefly, transformed yeast strains were grown overnight in SD-Ura to an OD_{600} = 2-4, washed once with water and resuspended in YPD (Yeast Peptone-Dextrose; 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose). After that they were incubated for an additional 1 hour at 30°C with shaking. Cells were washed twice with water and resuspended to an OD_{600} =10 in lyticase buffer (1.1 M sorbit, 20 mM Tris-HCl pH 7.6, 1 mM dithiothreitol (DTT)) containing 57 units of lyticase per ml.) After digestion (1.5-2 h), spheroblasts were collected by centrifugation (1200 x g, 10 min, RT). Cell pellets were then homogenized, on ice, in 30 ml of homogenization buffer [1.1 M glycerol, 50 mM Tris – ascorbate pH 7.4, 5 mM EDTA, 1 mM DTT, 1.5% (w/v) polyvinylpyrrolidone (PVP), 2 mg/ml bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer and 30 strokes with a tight-fitting glass piston. Two centrifugations (4000 x g, 10 min, 4°C) were used to remove unbroken cells and debris. The supernatants were collected, combined and centrifuged (100 000 x g, 45 min, 4ºC). The pellet containing microsomal membranes was resuspended to an OD_{600} = 4 in storage buffer (1.1 M glycerol, 50 mM Tris-MES pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF). Aliquots (250 µl) were immediately used for transport assays or stored at - 80ºC for further use.

Proton translocation across the vesicle membranes was determined by measuring the quenching and recovery of fluorescence of the ΔpH probe 9-amino-6-chloro-2-methoxyacridine (ACMA) (Gomez *et al*, 2009). The assay mixture (1 mL) contained transport buffer (0.4 M glycerol, 0.1 M KCl, 20 mM Tris-MES, 1 mM dithiothreitol, 6 mM $MgCl_2$, pH 7.4), 2 µM ACMA and 75 uL of yeast microsomes (corresponding to 300 µg of total protein). The fluorescence intensities of the probes were measured using a plate reader spectrophotometer (Fusion™ α, Packard) after the addition of 5 mM MgATP. At steady state, 0.2 mM cyanidin 3-glucoside was added and changes in fluorescence monitored. Fluorescence was measured at a wavelength of 485 nm, after excitation at 425 nm (15 sec). As a reference point the pH gradient in the assay mixture described above was abolished by addition of $(NH_4)_2SO_4$ to a final concentration of 25 mM.

4.3. RESULTS

4.3.1. ATDTX35

4.3.1.1. ATDTX35 IS TRANSCRIPTIONALLY UPREGULATED IN PAP1-D

Since anthocyanin synthesis is difficult to induce reproducibly, we produced an RNAi construct to downregulate *AtDTX35* in an Arabidopsis background overexpressing the MYB transcription factor-PAP1, which induces constitutively (anthocyanin hyper-accumulator). Interestingly, several lines carrying the RNAi construct exhibited a strong phenotype, where the red-color (anthocyanin hyper-accumulation) reverted to green (wild type state).

We performed semi-quantitative RT-PCR (sqPCR) analyses of the selected *AtDTX35* RNAi lines, wild-type (Col-0), the anthocyanin hyper-accumulator PAP1-D and a *dtx35* T-DNA mutant. Unexpectedly sqPCR indicated that in both selected lines key flavonoid biosynthetic genes (*PAL1*, *CHS*, *DFR*) showed reduced transcript abundance. *AtDTX35* transcript was not detected in the leaves of a T-DNA insertion line (Fig. 4.2).

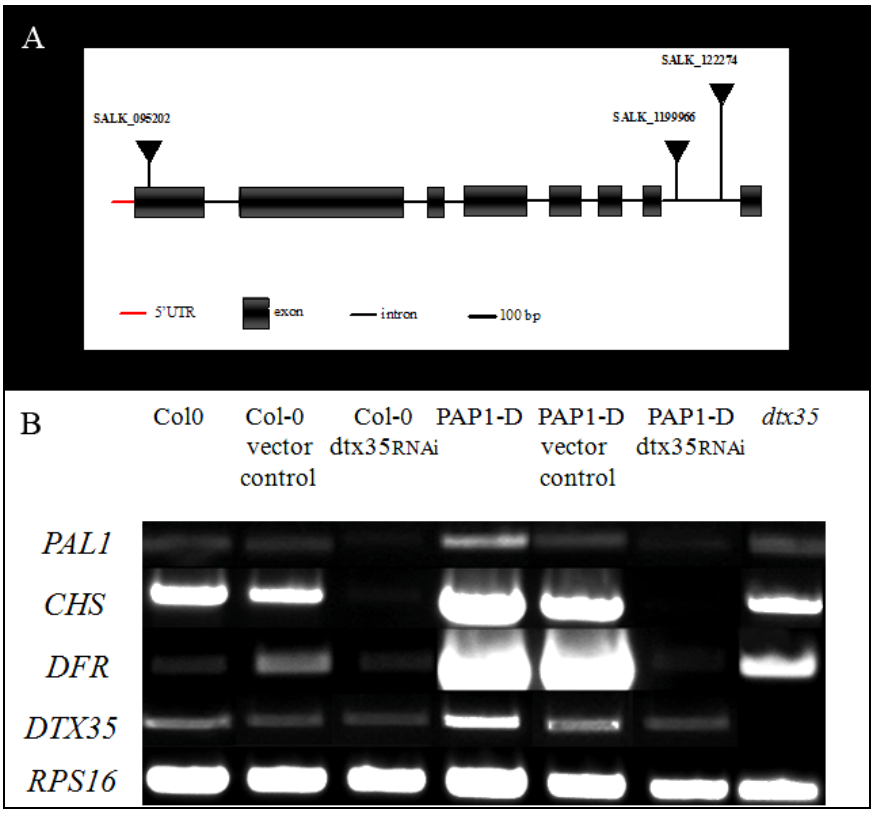


Figure 4.2. Genetic organisation of *atdtx35* T-DNA insertion mutants (A) and semi-quantitative reverse transcription (sqPCR) of *DTX35* gene expression in the leaves of Arabidopsis wild type plants (Col-0) and various flavonoid biosynthetic genes that hyper-accumulate anthocyanins (PAP1-D), PAP1-D expressing dtx35RNAi and *dtx35* loss-of-function mutant (B). PAL1 (phenylalanine ammonia-lyase, At2g37040) is an isoform of PAL family in Arabidopsis and catalyzes the first step in

the biosynthesis of phenylpropanoids; *CHS* (chalcone synthase, At4g00040) is a key enzyme of flavonoid biosynthesis; DFR (dihydroflavonol 4-reductase, At5g42800) is a key enzyme involved in anthocyanin biosynthesis which catalyses the reduction of dihydroflavonols to leucoanthocyanidins in the anthocyanin pathway; *RPS16A* (At2g0990) is a constitutively expressed 40S ribosomal gene. The lines expressing *dtx35$_{RNAi}$* were created as outlined in the materials and methods.

As previously shown (Tohge *et al*, 2005), we observed high transcript level of *CHS* in PAP1-D and PAP1-D transformed with an empty vector control but also in Col-0 and Col-0 transformed with an empty vector. PAP1-D and PAP1-D transformed with an empty vector control showed also high transcript level of *DFR*, which is normally upregulated in the PAP1-D (Tohge *et al*, 2005). PAP1-D transformed with dtx35$_{RNAi}$ construct showed low level or absence of transcript for all the key enzymes of biosynthesis of flavonoids which may suggest that silencing process of DTX35 might also silenced other genes and that this resulted in the change of coloration of PAP1-D (*DTX35* transcript in PAP1-D dtx35$_{RNAi}$ silenced line is low but present) (Fig. 4.2).

4.3.1.2. DTX35 DOES NOT COMPLEMENT SEED PHENOTYPE OF TT12

To investigate whether *DTX35* is able to complement *tt12* and restore its functionality, we cloned *DTX35* into a binary vector pMDC83Ban5pr (Curtis and Grossniklaus, 2003) and stably transformed *tt12* mutant plants using Agrobacterium. T1 plants were selected on ½ MS containing hygromycin B (25µg/ml) and dry, mature T2 seeds were used for complementation tests with DMACA, which is interacting with PAs forming red or black colored adducts visible as change in seed coloration from brown to red and black.

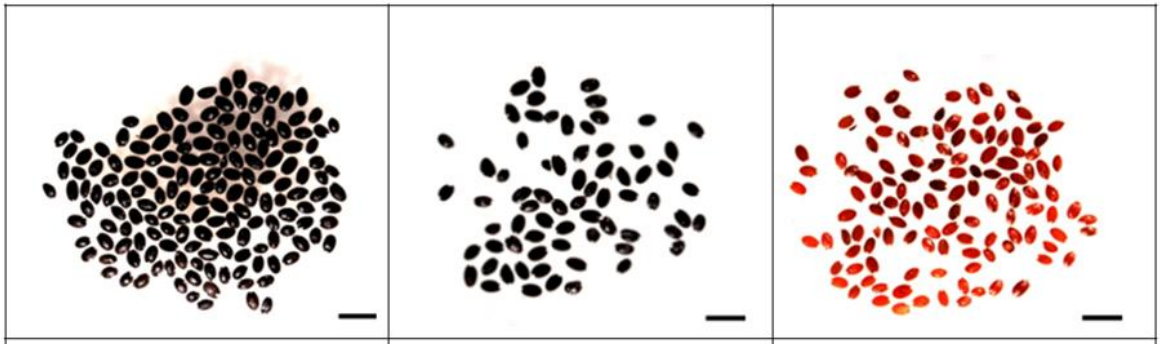


Figure 4.3. DMACA-staining of dried T2 seeds of *tt12* transformed with pMDC83Ban5pr:TT12 or pMDC83Ban5pr:DTX35. Presence of PAs in wild type (Col-0), ProBAN:TT12 in *tt12* and ProBAN:DTX35 in *tt12*. Bar = 1 mm

The DMACA staining of wild type Col-0 seeds produced black seeds. Similarly, TT12 under the control of BANYLUS promoter was able to complement *tt12* seed phenotype and resulted in black coloration. In contrast, T2 seeds of DTX35 under the control of BANYLUS promoter in *tt12* mutant showed red coloration which indicates that *DTX35* is unable to complement *tt12* seed phenotype (Fig. 4.3).

4.3.1.3. A DTX35 PROMOTER Β-GLUCORONIDASE FUSION SHOWS UBIQUITOUS PROMOTER ACTIVITY

In order to specify the activity of the *DTX35* promoter, we used 1.9 kb-genomic fragment corresponding to the putative DTX35 promoter region (Pro_{DTX35}) fused to the β-glucoronidase reporter gene. Plants were grown on full strength MS media containing 1% sucrose and 25µg/ml hygromycin B and then 7 d old seedlings were stained for GUS activity.

Figure 4.4. Activity of the DTX35 promoter in T2 plants transformed with Pro_{DTX35}*:GUS* construct. Bar = 1mm

Our analyses showed that DTX35 is expressed ubiquitously in the young seedlings (Fig. 4.4).

4.3.1.3. FLAVONOID PATTERN IS NOT MODIFIED IN DTX35$_{RNAi}$ SILENCED LINES

For the analysis of possible changes in flavonoid pattern amongst dtx35$_{RNAi}$ silenced lines, Col-0 and PAP1-D both transformed with an empty vector and dtx35RNAi construct, were grown in soil for 3-4 weeks. Leaves of all plants were separately pooled and analyzed. The metabolite analysis was performed by HPLC-RID.

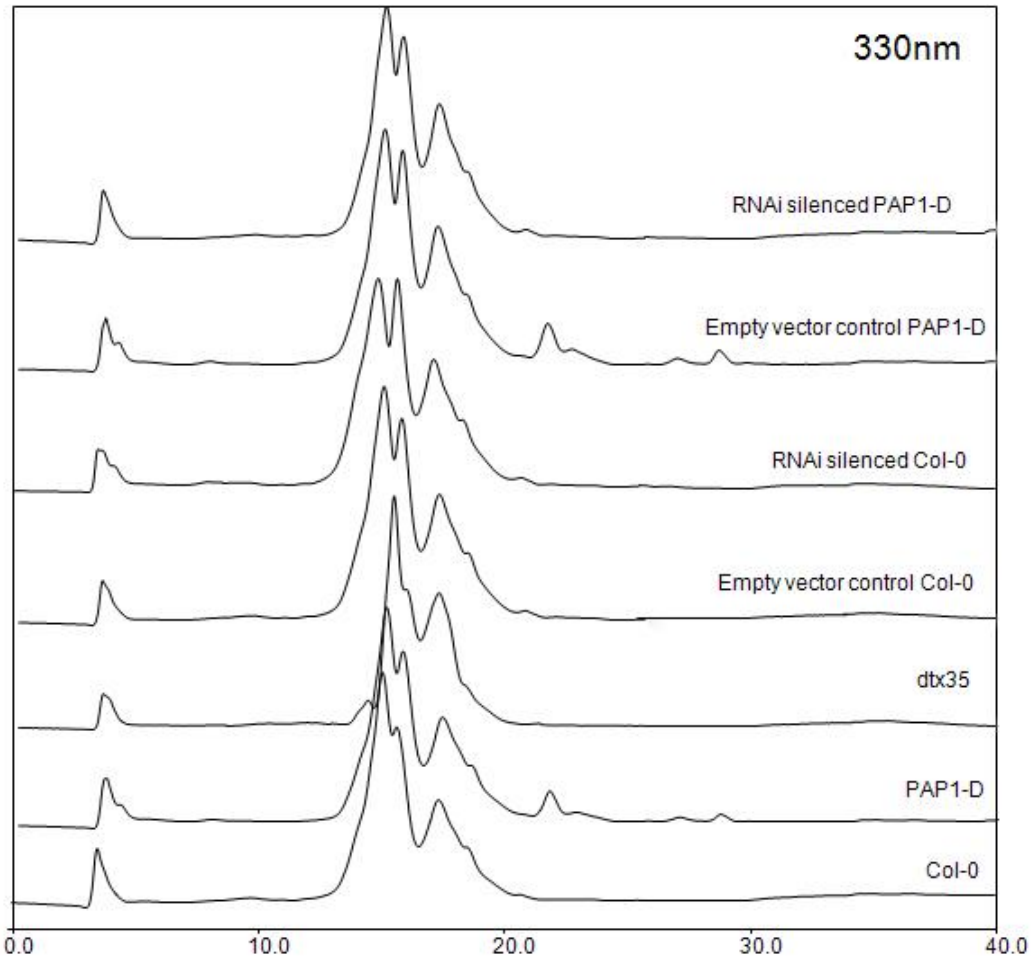


Figure 4.5. Flavonoid accumulation in $dtx35_{RNAi}$ silenced lines and the controls.

PAP1-D and PAP1-D transformed with an empty vector did show similar flavonoid pattern in the analysis. dtx35 RNAi silenced lines show similar profiles to Col-0 and dtx35 knock-out mutant (Fig. 4.5).

4.3.1.4. DTX35 DOES NOT TRANSPORT CYANIDIN-3-GLUCOSIDE

To examine the anthocyanin transporter function of AtDTX35, we performed the in vitro studies using ACMA, the pH sensitive fluorescent dye. We performed the proton pumping tests and demonstrated the physiological intactness of the yeast vesicles and that the addition of cyanidin-3-gluoside reduced the H^+- pumping into the yeast vesicles expressing DTX35 (Fig. 4.6).

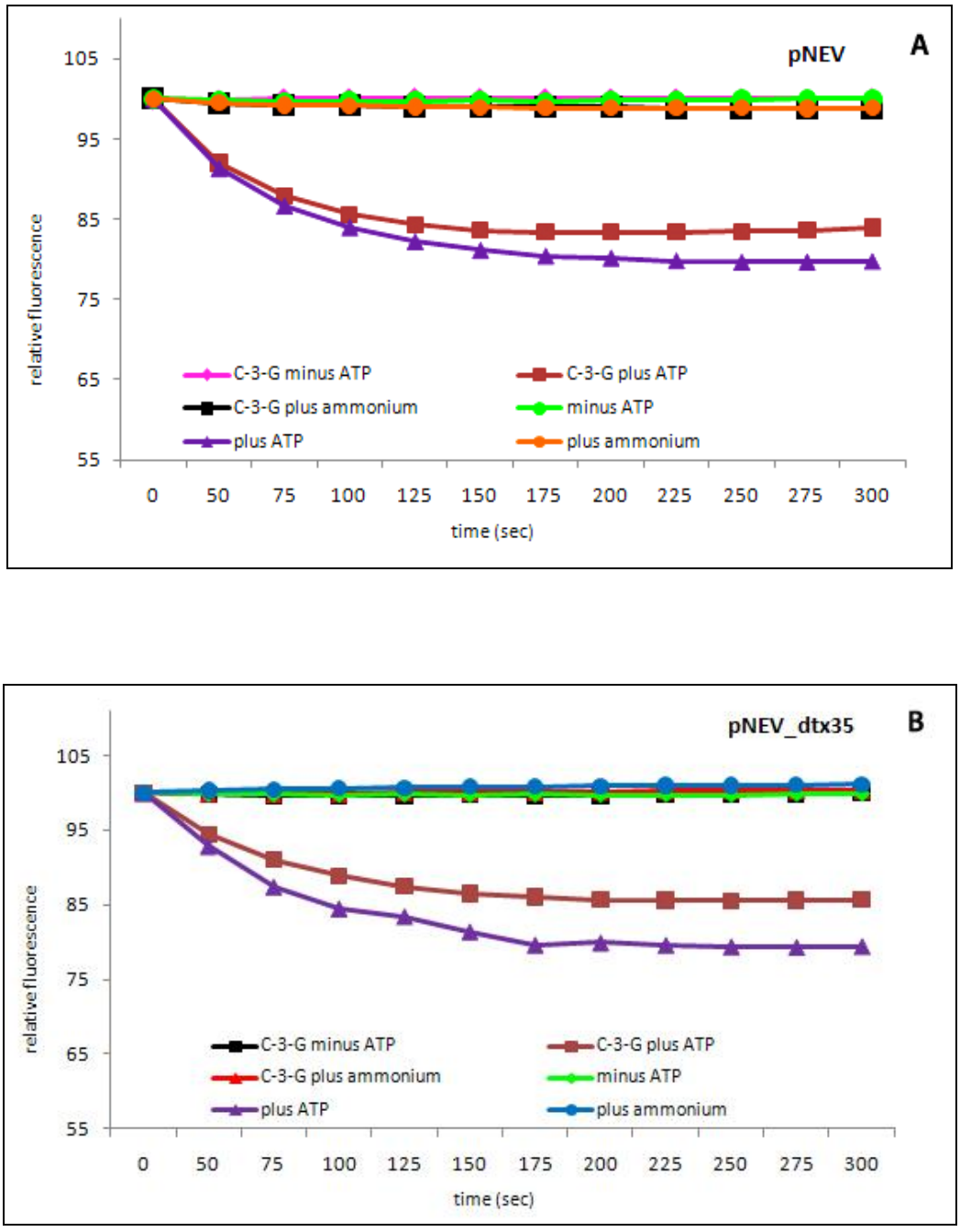


Figure 4.6. Cyanidin-3-glucoside does not reduce H^+- pumping in yeast vesicles expressing *DTX35*. Fluorescence quenching of the pH sensitive ACMA indicating ATP-dependent acidification of microsomes isolated from (A) DTX35 and (B) pNEV (empty vector) transformed yeast.

4.3.2. DTX31

4.3.2.1. ATDTX31 IS TRANSCRIPTIONALLY UPREGULATED IN ARABIDOPSIS ROOTS UNDER SALT STRESS CONDITIONS

We performed sqPCR analyses of gene expression in *in vitro* grown Arabidopsis (Col-0) and dtx31 T-DNA mutants grown on MS and subsequently transferred to MS supplemented with 75 mM NaCl. The results indicated that *AtDTX31* gene is expressed only in the roots of Col-0 plants subjected to the salt stress and the lack of transcript in the *dtx31* lines in the same conditions (Fig.4.7).

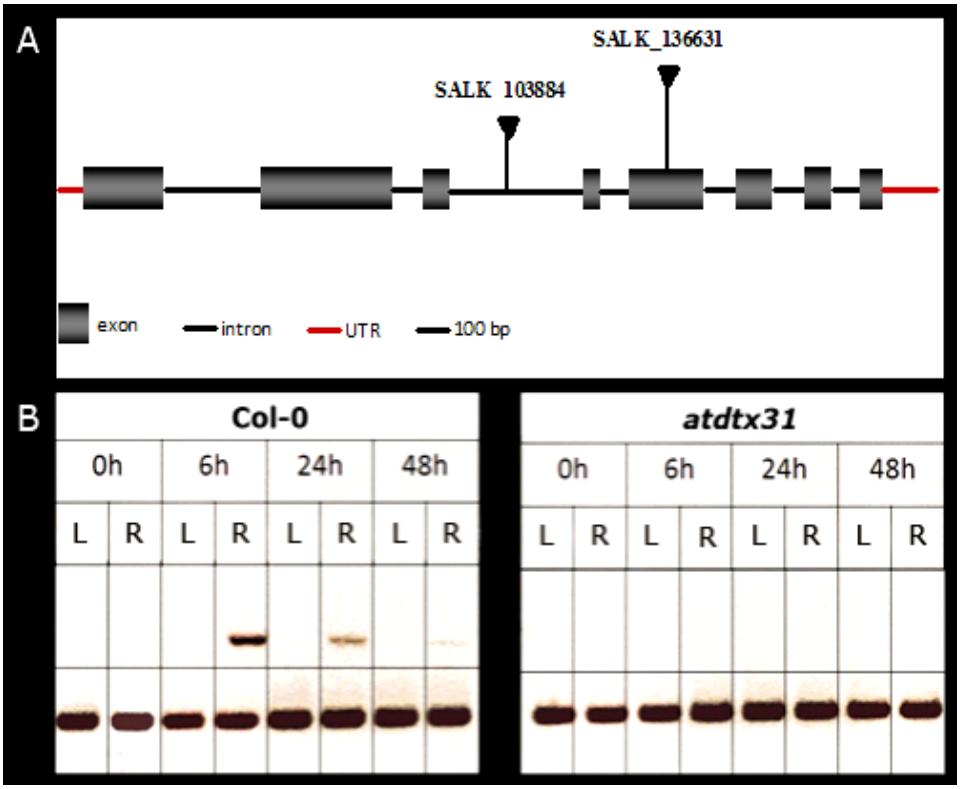


Figure 4.7. Characterization of *AtDTX31* gene (A) Structure of *DTX31* gene and positions of T-DNA insertions. (B) Semi-quantitative reverse transcription (sqPCR) of *DTX31* gene expression in the leaves and roots of Arabidopsis wild type plants (Col-0) and *atdtx31* knock-out mutant subjected to 75mM salt stress for 0, 6, 24 and 48h. L-leaves; R-roots

Interestingly, the highest expression level of DTX31 was observed after 6h of subjecting Col-0 plants to 75 mM NaCl and then it gradually decreased to 48 h of treatment.

4.3.2.2. ATDTX31 IS NOT EXPRESSED IN RESPONSE TO OSMOTIC STRESS

In order to examine whether the expression of AtDTX31 correlates to salt stress and not osmotic stress, we performed sqPCR analysis of gene expression in *in vitro* grown Arabidopsis Col-0 and dtx31 loss-of-function mutant plants grown on MS supplemented with 50 mM NaCl and 100 mM mannitol which characterize the same osmolality.

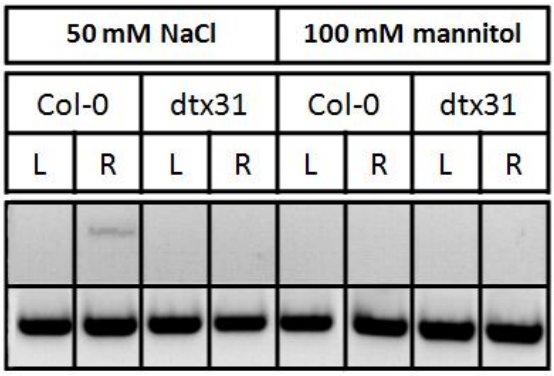


Figure 4.8. Semi-quantitative reverse transcription PCR analysis of DTX31 gene expression in the leaves and roots of Col-0 and dtx31 knock-out mutant subjected to 50 mM NaCl and 100 mM mannitol. L-leaves; R-roots

We observed that AtDTX31 is expressed only in the roots of Col-0 under the salt stress and not under the osmotic stress (Fig. 4.8). Dtx31 knock-out mutant did not show any expression which confirms previous results from chapter 4.3.2.1.

4.3.2.3. PHENOTYPIC ANALYSES OF DTX31 IN RESPONSE TO SIMPLE PHENOLICS

To investigate the involvement of AtDTX31 in flavonoid biosynthesis, Col-0 and dtx31 knock-out mutant plants were grown on ½ MS medium for 10 days and then transferred to the ½ MS supplemented with simple phenolics (0.5mM cinnamic acid, 100 nM quercetin, 200 nm quercetin) for 5 days. Under these conditions, we did not observe any differences in between Col-0 and dtx31 mutant growth or any other phenotypic changes (Figure 4.9).

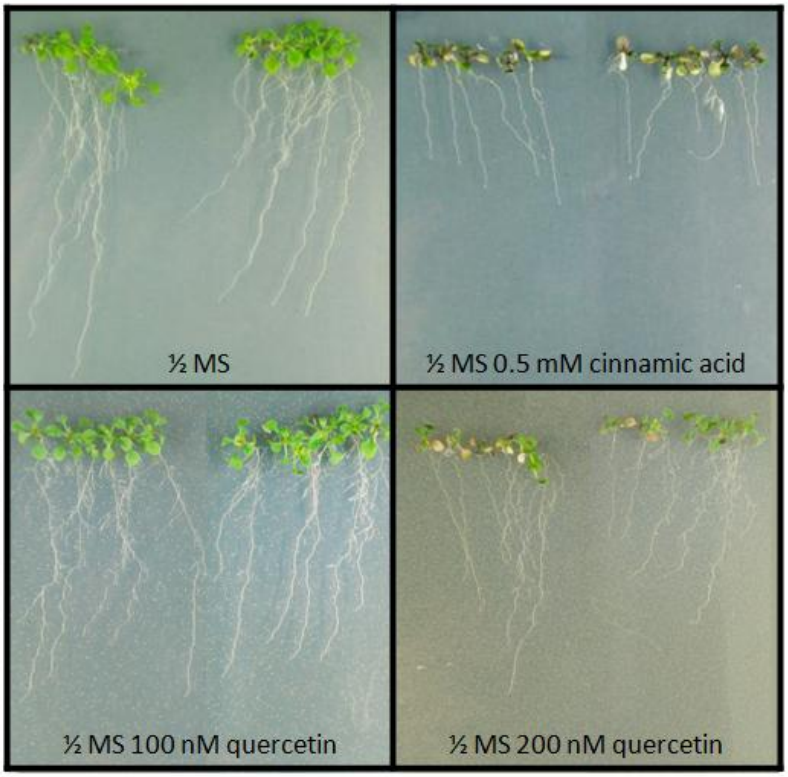


Figure 4.9. Plate tests of transgenic plants grown on ½ MS containing simple phenolics. Right-wild-type, left-*dtx31* knock out mutant

4.3.2.4. DTX31-PROMOTER-GUS STUDIES SHOW SPATIAL ACTIVITY OF THE DTX31 PROMOTER UNDER THE SALT STRESS

To examine the tissue specific activity of DTX31 promoter we cloned the construct where 1.2 kb DNA fragment upstream of start codon was fused to a β-glucoronidase reporter gene. Plants were grown on full strength MS medium containing 1% sucrose and 25 µg/ml of hygromycin B. Selected plantlets were then transferred to MS plates supplemented with 150 mM NaCl and stained for GUS activity after 24, 48 and 72 h. We observed spatial activity of the DTX31 promoter in response to salt treatment (Fig. 4.10).

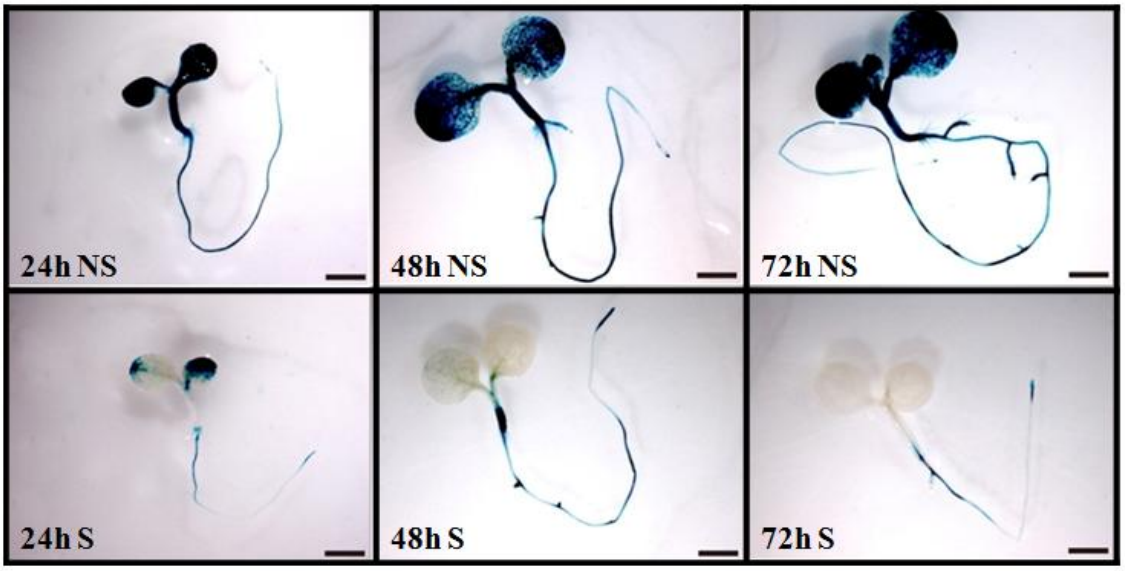


Figure 4.10. Activity of the DTX31 promoter in the seedlings of transgenic Arabidopsis. NS-non stressed, S-stressed, Bar = 1 mm

When the roots of these plantlets were cross sectioned using Technowit 710, we observed that DTX31 promoter activity was restricted to the vascular bundle and pericycle (Fig. 4.11).

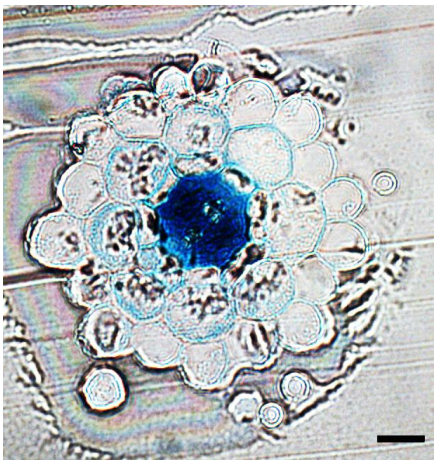


Figure 4.11. Cross section of *atdtx31* root subjected to salt stress. Bar = 75 µm

4.3.2.5. DTX31 IS LOCALIZED TO THE TONOPLAST

To investigate the subcellular localization of DTX31, the full-length *DTX31* cDNA was fused to the coding sequence of *GFP*, resulting in C-terminal fusion of green fluorescent protein to DTX31. Expression of this fusion was driven by the dual cauliflower mosaic virus 35S RNA promoter and

analyzed after transient expression in *Nicotiana benthamiana* via *Agrobacterium tumefaciens* infiltration followed by isolation of mesophyl protoplasts. The GFP signal was restricted to a vacuolar membrane (Fig. 4.12. upper panel). Gentle lysis of the mesophyl protoplasts demonstrated clearly that the GFP fluorescence localizes to the tonoplast (Fig. 4.12. lower panel).

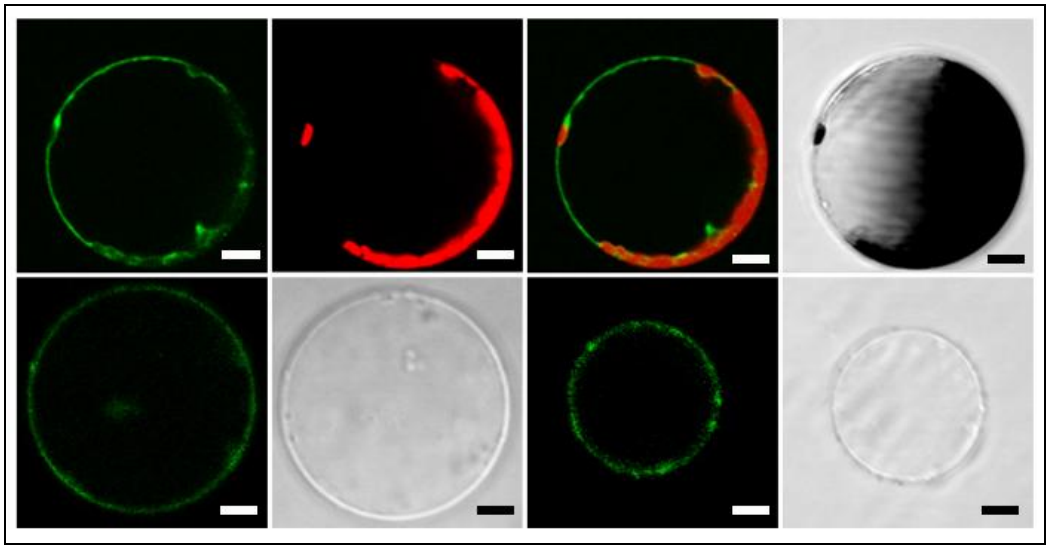


Figure 4.12. Subcellular localization of the DTX31 protein after transient expression. Upper panel: Protoplasts isolated from the mesophyl of transiently transformed plants. Lower panel: The vacuoles released by gentle lysis, exhibiting GFP signal on the tonoplast. Bar = 50 µm

4.3.2.5. DTX31 LOSS-OF-FUNCTION MUTANT DOES NOT SHOW AN AGRAVITROPIC RESPONSE TO A SALT STRESS

In order to examine whether DTX31 is involved in gravitropic response of the roots to salt stress, Col-0 and dtx31 mutant plants were grown vertically on full-strength MS media enriched with 3% sucrose, then transferred to a salt-containing MS media and grown inverted for 7-10 days. Arabidopsis grows relatively normally at 50 mM NaCl but concentrations higher than 150 mM NaCl are toxic. Figure 4.13 shows no differences in root bending between Col-0 and dtx31 knock-out mutant. It was also observed that both Col-0 and dtx31 mutant grow slower on MS supplemented with 150 mM NaCl compared to MS without salt but no differences in growth rates were observed between Col-0 and dtx31.

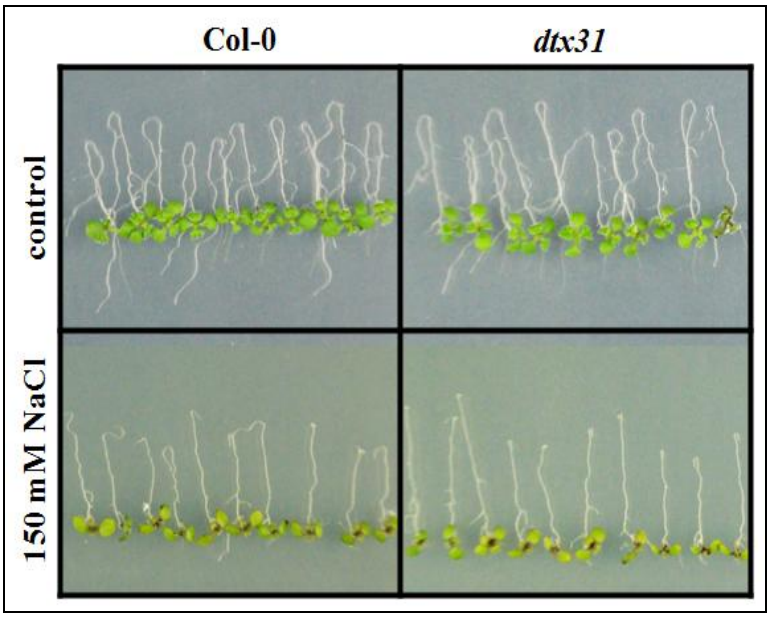


Figure 4.13. Root-bending assay performed on Col-0 and dtx31 knock-out mutant**.**

4.2.3.7. HPLC-RID ANALYSES SHOW A SIGNIFICANT ALTERATION IN THE FLAVONOID PATTERN

We performed HPLC-RID analyses on wild-type Col-0 and dtx31 knock-out lines, which were grown hydroponically in standard Arabidopsis medium (see chapter 4.2.1) and then transferred to the medium containing salt (75 mM NaCl). Root exudates were collected after 6, 24 and 48 h of treatment and HPLC samples were prepared as described in chapter 4.2.6.8.1.2.

We observed that there are significant alterations in the flavonoid pattern between Col-0 and dtx31 knock-out mutant and also the appearance of unidentified metabolites after 24h in dtx31 metabolic profile when compared to the sample from 6h and 48h. We did not detect similar metabolic profile after 24h for the wild-type (Fig. 4.14).

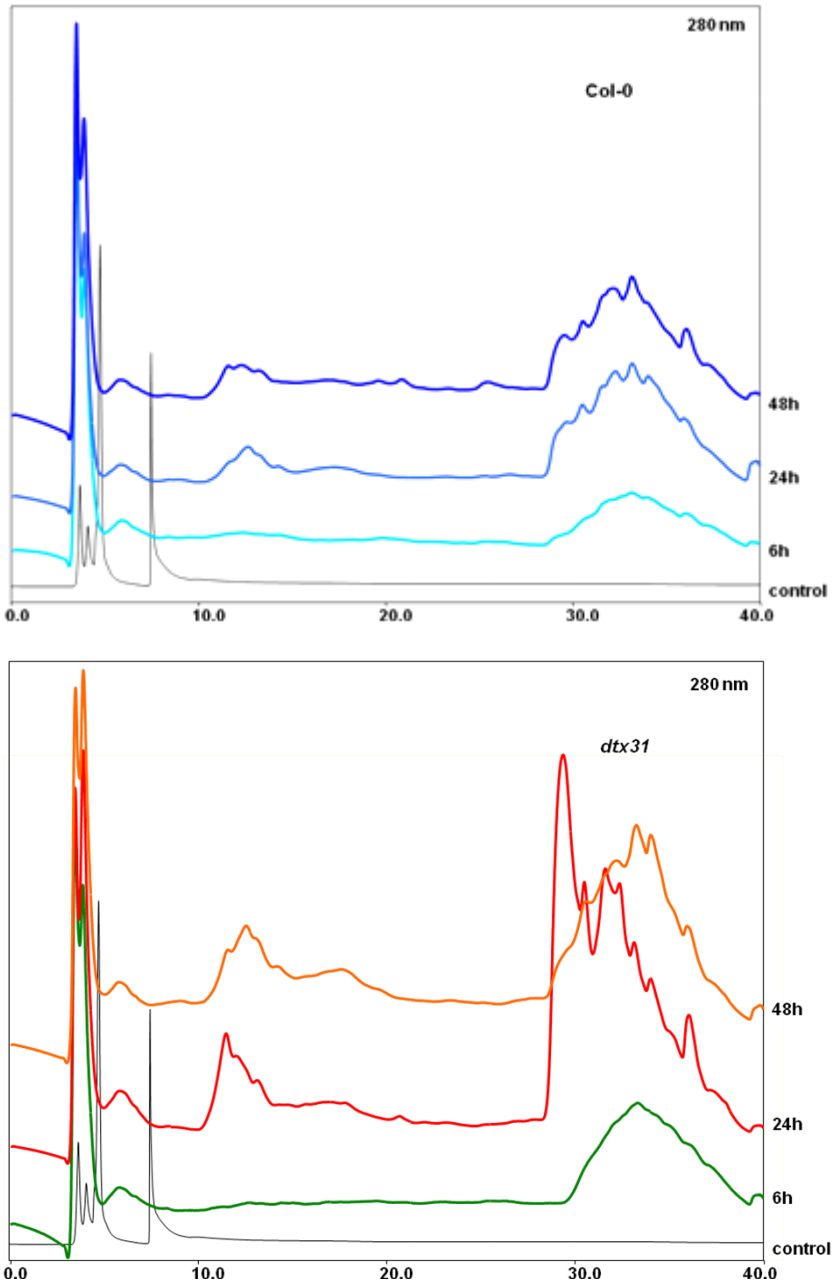


Figure 4.14. HPLC-RID analyses of the root exudates from hydroponically grown Col-0 and *dtx31* plants subjected to a salt stress (75 mM) for 6, 24 and 48 hours. Control samples represent only hydroponic medium with 75 mM NaCl.

4.3.3. DTX28

4.3.3.1. DTX28 DOES NOT SHOW HIGHER EXPRESSION LEVEL IN MYB12 OVEREXPRESSING LINE

To obtain homozygous dtx28 T-DNA insertion line, plants were grown in soil for 3-4 weeks, the leaves of single plants were collected and genomic DNA was isolated. This DNA was used for PCR analysis with 7 combinations of primers (Fig. 4.15.B) and the homozygous lines were selected.

To verify whether DTX28 is involved in the flavonol biosynthesis, we performed semi-quantitative reverse transcriptase PCR on Col-0, MYB12 ox, myb12 knock-out and myb11myb12myb111 triple knock-out plants grown for 14 d on ½ MS supplemented with 1% sucrose. The results show that there are no significant differences between transcript levels of aforementioned lines. The expression level of *DTX28* appears to be lower in MYB12 overexpressing and knock-out lines compare to triple knock-out mutant and Col-0.

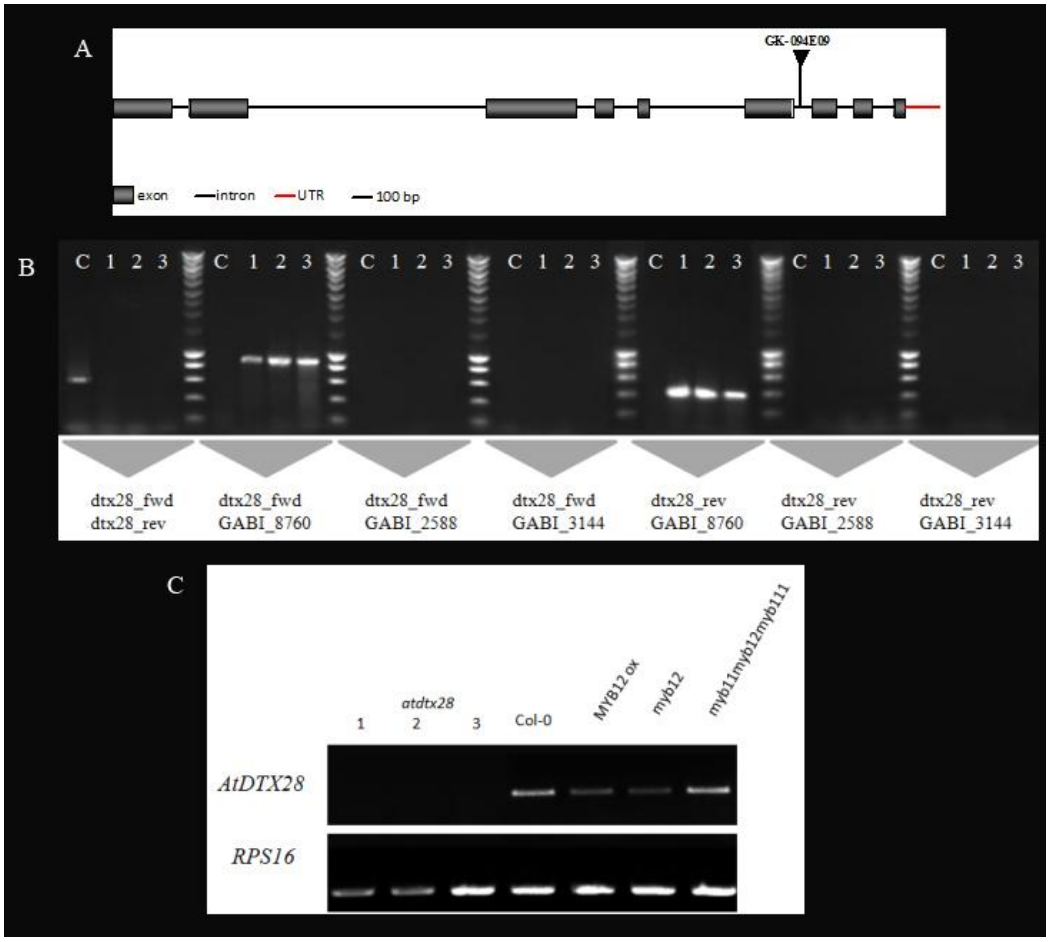


Figure 4.15. Characterization of *AtDTX28* gene (A) Structure of *DTX28* gene and position of T-DNA insertion; (B) Homozygosity screen of GABI KAT line GK-094E09 with different

combinations of primers (as designated under the gel picture); (C) Semi-quantitative reverse transcription PCR (sqPCR) of *DTX28* gene expression in the seedlings of Arabidopsis wild type plants (Col-0) and *atdtx28* knock-out mutants and *MYB12* overexpressing line, *myb12* knock-out and triple knock-out *myb11myb12myb111* plants.

In order to ensure that the homozygous knock-out lines lack the *DTX28* gene transcript, we performed sqRT-PCR on pre-screened plants grown for 14 d on ½ MS supplemented with 1% sucrose. The results confirmed that three pre-selected lines are homozygous knock-out mutants for *DTX28* gene (Fig. 4.15 C) .

3.3.3.2. DTX28 PROMOTER FUSED TO B-GLUCORONIDASE REPORTER GENE SHOWS UBIQUITOUS PROMOTER ACTIVITY

To study the tissue specific activity of DTX28 promoter we cloned the construct where 0.8 kb DNA fragment upstream of start codon was fused to a β-glucoronidase reporter gene. Transformed plants were grown on full strength MS medium containing 1% sucrose and 25 µg/ml of hygromycin B and then stained for GUS activity according to the protocol (Fig. 4.16).

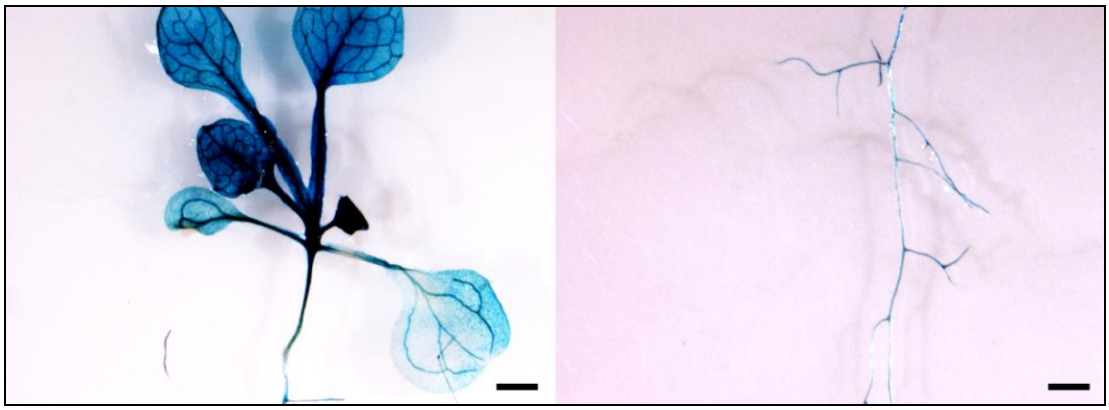


Figure 4.16. GUS-DTX28 promoter activity in transgenic Arabidopsis. Bar = 1 mm

The GUS staining analysis showed that DTX28 promoter is expressed ubiquitously.

4.3.3.3. SUBCELLULAR LOCALIZATION OF DTX28

To examine the subcellular localization of DTX28, the full-length cDNA was fused to GFP on C-terminus and transiently expressed in *N. benthamiana* by infiltration with *A. tumefaciens* GV3101

carrying. The infiltration was followed by isolation of mesophyl protoplasts and confocal-scanning microscopy analysis.

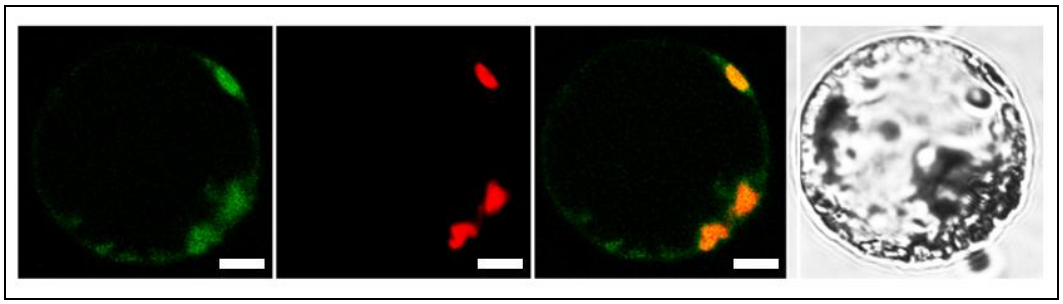


Figure 4.17. Subcellular localization of DTX28 in protoplasts of transiently transformed *N. benthamiana* via infiltration with *cDTX28-GFP* construct. Bar = 50 µm

Confocal microscopy analysis of protoplasts indicates that DTX28 localizes to the plasma membrane (Fig. 4.17). However, further analyses have to be performed to verify this preliminary data.

4.3.3.4. HPLC-RID ANALYSES OF DTX28 KNOCK-OUT LINES

In order to verify whether there is an alteration in flavonoid patterns between the AtDTX28 loss-of-function mutant and wild type plants, plants were grown on soil for 3-4 weeks, leaves were collected and prepared for the analysis as described in chapter 4.2.6.8.1.1 HPLC-RID assay was performed as shown in chapter 4.2.6.8.

HPLC-RID analyses of the seedlings of wild-type and dtx28 knock-out plants subjected shows no significant alteration in the flavonoid pattern between Col-0 and the *dtx28* mutants (Fig. 4.18).

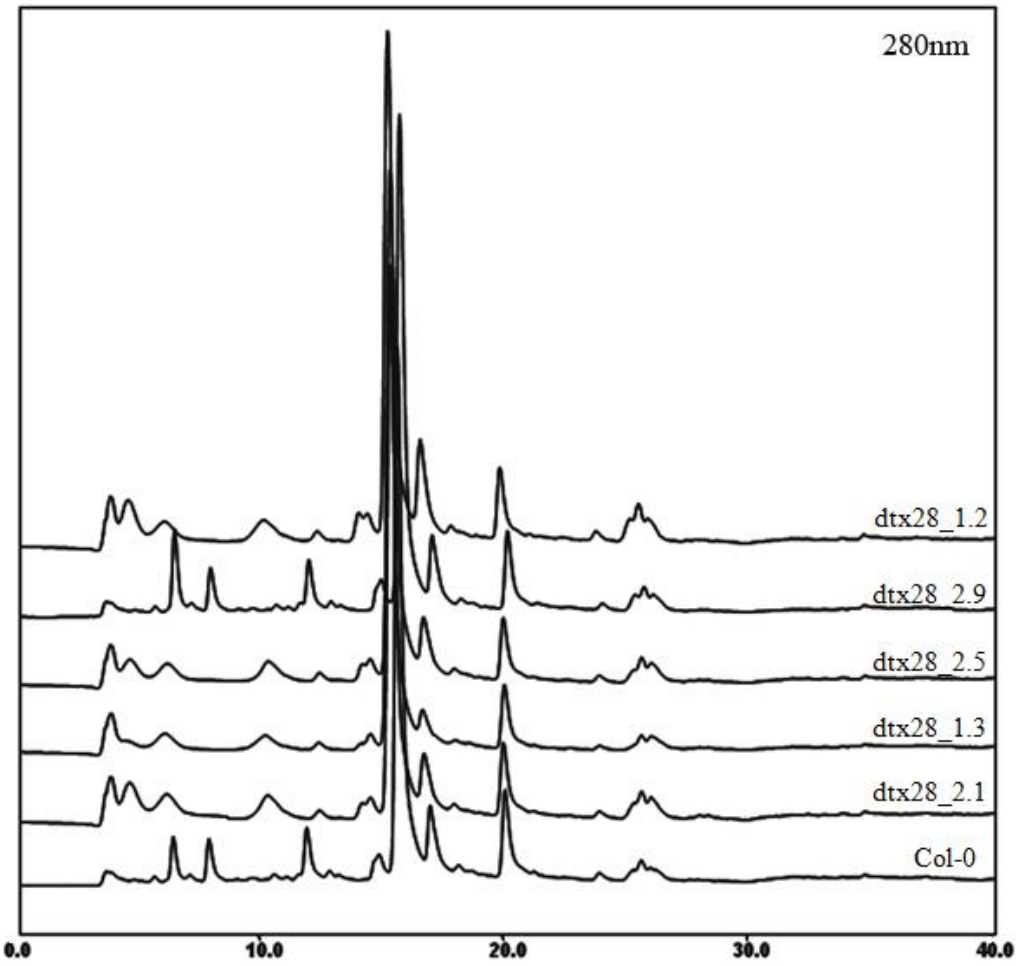


Figure 4.18. HPLC-RID analysis of wild-type and DTX28 knock-out plants.

4.4. DISCUSSION

Flavonoid transport plays essential roles in plant development, growth, reproduction and defense. Their transport from the site of synthesis (mainly cytosol) to the correct cell compartment and between tissues is crucial and complex (Buer *et al*, 2007). As described previously (Zhao and Dixon, 2010), there are three known mechanisms of flavonoid transport into the vacuole: vesicle trafficking, GST-linked and MATE transporters, although in the case of the latter, knockout protein studies involved in transport of proanthocyanidins such as TT12, MtMATE1 or TT19 still detect low level of PAs in the seed coat, which suggests the existence of additional mechanisms involved in PA sequestration into the vacuole (Marinova *et al*, 2007; Zhao and Dixon, 2009 Kitamura *et al*, 2004a). This work was aimed to elucidate biochemical and molecular features of transport steps involved in the flavonoid accumulation in Arabidopsis.

Based on published data we selected and investigated three candidate genes encoding putative flavonoid transporters in Arabidopsis (i) DTX35 (At4g25640), (ii) DTX31 (At1g12950) and (iii) DTX28 (At5g44050).

4.4.1. AtDTX35 AS A PUTATIVE ANTHOCYANIN TRANSPORTER

AtDTX35 shows high homology to a tomato MATE which exhibits high expression in the *ANT1* mutant. *ANT1* mutant was isolated by screening activation-tagged mutants and was characterized by deep purple pigmentation of the whole plant (hyperaccumulation of anthocyanins). *ANT1* encodes a MYB transcription factor and its activation and/or overexpression resulted in the coordinated upregulation of the expression of some flavonoids genes such as *CHS* or *DFR* and transporter-like genes defined as *MPT77* (Mathews *et al*, 2003). *MTP77* is highly homologous to Arabidopsis *TT12* and encodes most likely candidate for an anthocyanin transporter in tomato.

Our mutant characterization approach was started by producing an RNAi contruct to downregulate *AtDTX35*. Since pigmentation in wild-type Arabidopsis is very variable, we used Arabidopsis line overexpressing PAP1 MYB (Ox-*PAP1*) transcription factor, which results in the red-coloured plants due to hyperaccumulation of anythocyanins. Interestingly, several lines (Ox-*PAP1* background) carrying the RNAi construct exhibited a strong phenotype, where the red-color (anthocyanin hyper-accumulation) reverted to green (wild-type state). We performed semi-quantitative RT-PCR (sqPCR) analyses of the selected Ox-*PAP1*/*AtDTX35*$_{RNAi}$ lines, wild-type (Col-0), the anthocyanin hyper-accumulator Ox-*PAP1* and a *dtx35* T-DNA mutant. Unexpectedly sqPCR indicated that in Ox-PAP1/ dtx35$_{RNAi}$ silenced lines, key flavonoid biosynthetic genes (*PAL1*, *CHS*, *DFR*) showed reduced transcript abundance (Fig. 4.2). We concluded that silencing of DTX35 in PAP1 background led to downregulation of all key enzymes genes which are normally upregulated in the Ox-PAP1 background (Borevitz *et al*, 2000, Tohge *et al*, 2005), therefore we hypothesized that the change in coloration was most likely a result of the downregulation of these genes and not necessarily due to the silencing of DTX35. Moreover, mutations in the CHS, DFR and LDOX genes cause the reduction in the pigmentation of plants (Shirley *et al*, 1995 Abrahams *et al*, 2003), suggesting that these proteins are necessary for the synthesis of anthocyanidin and proanthocanidin. The promoter regions of these flavonoid biosynthetic genes contain MYB-binding sites (Nesi *et al*, 2001; Debeaujon *et al*, 2003), consequently the observed pleiotropic effect might be the result of the downregulation of these genes.

To confirm that silencing did not change flavonoid pattern of mutant lines compared to the wild-type and control lines, we performed HPLC analyses. It was expected to detect changes in flavonoid accumulation due to the change in coloration. Particularly, because our sqPCR detected silencing in DFR (Fig. 4.2), which is a crucial protein facilitating anthocyanin pigmentation in lettuce (*Lactuca sativa*) and repressing this protein would result in green leaf phenotype (Park *et al*, 2008).

Our analysis demonstrated that the DTX35 downregulation altered flavonoid pattern of Ox-PAP1 lines where the silencing construct was incorporated into the genome. Col-0, dtx35 knock-out mutant and RNAi silenced lines showed similar flavonoid profiles, indicating that our silencing experiment modified the flavonoid biosynthesis genes (Fig. 4.5).

To validate our hypothesis that *AtDTX35* is a putative anthocyanin transporter we used the misexpression strategy aiming at the expression of DTX35 gene in the background of *tt12*. We assumed that genes reverting the seed phenotype of *tt12* are promising candidates to be transporters for PA-related flavonoids (e.g. anthocyanins) in vegetative plants parts. We transformed *tt12* mutant with *DTX35* under the control of BANYLUS promoter. Subsequently, we stained dry T2 seeds of transgenic lines with DMACA which is used to detect presence of PAs in the seed coat. We observed that DTX35 was unable to complement tt12 seed phenotype (Fig 4.3), which suggests that DTX35 might not be involved in the transport of PA-related flavonoids.

Recent studies (Thompson *et al*, 2010) demonstrated that DTX35 is highly expressed in floral organs and to some degree in most tissues, including young seedlings, senescent leaves and roots. We partially confirmed this result in our GUS assay where we checked DTX35 promoter tissue specific activity by fusing it to β-glucoronidase reporter gene and observed that DTX35 promoter is active ubiquitously in young seedlings (Fig. 4.4). It has been also presented that this gene is involved in flavonols rather than anthocyanin transport. *In vitro* transport experiments with yeast expressing DTX35 with ACMA, a pH sensitive fluorescent dye, demonstrated that cyanidin 3-glucoside does not reduce H^+- pumping, similarly to yeast transformed with an empty vector (pNEV) as a control (Fig 4.6). This suggests that our hypothesis that DTX35 is involved in anthocyanin transport was incorrect and confirms the previous studies (Thompson *et al*, 2010). Further studies should involve transport experiments with other flavonoid classes e.g. flavonols.

4.4.2. ATDTX31, A MATE PROTEIN HIGHLY EXPRESSED DURING SALT STRESS

Exposure to high level of NaCl affects plants and creates ionic stress in the form of the cellular accumulation of Cl^- and Na^+ ions. Conversely, salt stress influences also the homeostasis of other ions such as Ca^{2+}, K^+ and NO_3^- and therefore entails the analysis of how transport and compartmentation of these nutrients is altered during salinity stress (Maathuis, 2006). AtDTX31, amongst two other MATE transporters, has been demonstrated to be 10 fold upregulated in response to high salt concentrations and a potential candidate for a transporter involved in salinity stress (Maathuis, 2006).

In order to confirm that *AtDTX31* is expressed in salt stress conditions, we performed sqPCR analyses on leaves and roots of seedlings grown hydroponically on a hydropnic medium supplemented with 75 mM NaCl over 6, 24 and 48h. Our experiment confirmed that *DTX31* gene is

transcriptionally upregulated in wild-type Arabidopsis plants and its expression is specific only to roots. We did not detect any transcript in the leaves and in *dtx31* knock-out mutant (Fig. 4.7).

In areas of high salinity the osmotic potential in plants is dramatically altered, leading to osmotic stress and possible injury from salt stress. One of the ways in which plants are able to cope with high levels of osmotic potential is to store sodium in vacuoles; this process results in a lowering of water potential and a lowering of stress and the other way is to alter osmotic potential through osmolytes (Nuccio *et al*, 1999). We performed sqPCR analysis on plants grown on 50mM NaCl and 100 mM mannitol (concentrations represent the same osomolality level) to investigate whether *DTX31* gene shows high expression level due to the salt stress or due to osmotic stress. Our results indicated that *AtDTX31* transcript is expressed only if the plants are subjected to the salt treatment (Fig. 4.8).

It has been demonstrated that abiotic stress causes an increase in reactive oxygen species (ROS) production such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH) and singlet oxygen ($^1O_2$), which plants are able to effectively neutralize during normal aerobic metabolism (Xiong and Zhu, 2002). Too big amounts of highly reactive ROS can damage proteins, lipids and nucleic acids by the oxidation (Halliwell and Guteridge, 1985). Therefore plants are able to produce antioxidant compounds such as phenolic compounds, tocopherols, glutathione and carotenoids, which are effective in eliminating ROS. We decided to test *AtDTX31* as a putative transporter of simple phenolics by growing Col-0 and dtx31 knock-out mutant plants on ½ MS supplemented with cinnamic acid and quercetin. The lack of phenotypic changes between Col-0 and *dtx31* mutant suggests that *DTX31* is not involved in the transport of simple phenolic compounds in response to salt stress in Arabidopsis (Fig. 4.9).

The semi-quantitative analyses of DTX31 transcript revealed that the gene is only expressed in the roots. In order to examine whether DTX31 promoter activity will be also tissue specific, we fused the DTX31 promoter to β-glucoronidase reporter gene and transformed Col-0 plants with this construct. Plants were first grown for 7 d on ½ MS with 25 μg higromycin and then the selected one were transfered to ½ MS and ½ MS supplemented with 150 mM NaCl and stained for GUS activity after 24, 48 and 72 h. We observed spatial activity of DTX31 promoter (Fig. 4.10) in response to salt treatment over time. The leaf-GUS activity observed in unstressed transgenic plants under normal growing conditions (Fig. 4.10) may point to additional promoter elements (e.g. first exon) that were not cloned in the original 1.2 kB promoter sequence upstream of the DTX31 start codon. This also might be a result of the influence from flanking DNA sequences (van Leeuwen *et al*, 2001) or the presence of *cis* regulatory elements in 5'UTR (Wray, 2007).

Another step in our characterization of DTX31 gene was to obtain its localization in the cell. We performed the transient expression analyses by infiltrating *N. benthamiana* with *Agrobacterium* carrying *DTX31* gene fused to C-terminus of GFP protein. We assumed that DTX31 gene will localize either to the plasma membrane or to the tonoplast. Both plasma membrane and tonoplast localizations

would imply the involvement of *DTX31* in conferring salt tolerance in Arabidopsis as it was shown for a putative cation/proton antiporter from soybean cloned into Arabidopsis (Luo *et al*, 2005) and by overexpression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis* (Apse *et al*, 1999), respectively. *cDXT31-GFP* analyses demonstrated the localisation to the tonoplast (Fig. 4.12). The observed tonoplastic localization of AtDTX31 implies that this protein might be involved in the salt tolerance (Apse *et al*, 1999).

Flavonoids are known to negatively regulate auxin transport and affect root gravitropic response (Santelia *et al*, 2008). There have been studies conducted on genes increasing salt tolerance in transgenic Arabidopsis which led to the discovery of salt overly sensitive (SOS) mutants (Wu *et al*, 1996). These mutants exhibit agravitropic responses under salt stress. We hypothesized that since *DTX31* was clearly only expressed in roots during NaCl stress and if it was a flavonoid transporter, then loss-of-function mutants may show agravitropic response under NaCl stress which would suggest the involvement of *DTX3*1 indirectly in auxin transport in response to salt stress by transporting flavonoids into the root tip. We did not observe any difference in the hooking of the root tips of Col-0 and dtx31 mutants subjected to a gravistimulus while under salt stress. Our results indirectly suggest that *DTX31* is not required for the correct transport of auxin into the root tip in gravitropic response (Fig. 4.13).

The main functions of roots are the absorption of water and nutrients, the physical anchoring of plants in the soil and the secretion of active biochemicals which help roots penetrate the soil (Iijima *et al*, 2003) and regulate rhizosphere interactions. Root exudates contain the range of primary and secondary metabolites, proteins and peptides (Bais *et al*, 2008; Weisskopf *et al*, 2006). The spectrum of exuded metabolites is influenced by environmental factors and by the plant's gene repertoire. In order to investigate whether the roots of the dtx31 knock-out mutant compared to the wild-type subjected to the salt stress exhibit different exudates profiles, we conducted the hydroponics experiment where plants were grown in hydroponic media supplemented with 75 mM NaCl and the samples were collected after 6, 24 and 48h. We observed significant alteration in the metabolic profile of dtx31 knock-out mutant after 24h of salt treatment (Fig. 4.14). We did not detect this alteration in the wild-type chromatogram. This may suggest that dtx31 knock-out is releasing significant amount of secondary metabolites into the rhizosphere in response to high salt concentrations to compensate the lack of the gene.

Further characterisation of *AtDXT31* gene will involve (i) further salt experiments in hydroponic medium to determine the exact composition of root exudates and identify the compounds for Col-0 and *dtx31* knock-out mutants via HPLC-ESI-MS analysis in collaboration with Dr. Laurent Bigler (University of Zürich, Switzerland) and (ii) transport experiments with yeast microsomes carrying heterologously expressed DTX31 with identified secondary metabolites to confirm the role of DTX31; (iii) confirming the salt dependent changes in exudates composition for second T-DNA insertion line.

4.4.3. AtDTX28 AS A PUTATIVE FLAVONOL TRANSPORTER

UV-B radiation is not only a stress signal but also an environmental stimulus to direct plant growth and development including hypocotyl growth inhibition, flavonoid accumulation and changes in specific gene expression (Ulm and Nagy, 2005). *AtDTX28* has been reported to be induced after UV-B treatment in wild-type plants but not in the photomorphogenesis mutants *cop1* (*CONSTITUTIVELY PHOTOMORPHOGENIC1*) and *hy5* (*ELONGATED HYPOCOTYL5*) (Oravecz *et al*, 2006). It has been also demonstrated that increased transcript abundance of genes involved in flavonol biosynthesis and *AtMYB12*, which is a specific activator of flavonol biosynthesis is linked to the UV-B dependent, *cop1* and *hy5*-blocked induction of *DTX28* expression (Mehrtens *et al*, 2005). As a result we hypothesized that *DTX28* encodes a vacuolar flavonol transporter.

AtMYB12 is a flavonol-specific regulator of flavonoid biosynthesis. Recent studies of *myb12* mutant plants and MYB12 overexpressing plants demonstrated that there is a tight correlation between the expression level of MYB12 and the flavonol content of young seedlings. The triple mutant *myb11myb12myb111* does not accumulate flavonol glycosides but does accumulate anthocyanins, confirming the role of these three TFs only in flavonol accumulation (Stracke *et al*, 2007). We examined the transcript abundance level in *myb12*, MYB12-OX and *myb11myb12myb111* mutants by sqPCR to investigate whether *DTX28* will be upregulated in the MYB12-OX line and downregulated in knock-out lines (Fig. 4.15C). Our analyses did not confirm our hypothesis. Only Col-0 and triple-knockout mutant plants showed high transcript level. In both, *myb12* and MYB12-OX the *DTX28* appear to be downregulated.

To validate the results of sqPCR, we carried out HPLC-RID analyses on confirmed *dtx28* knock-out mutants and wild-type plants. We observed minor changes in the beginning of the HPLC runs in knock-out samples compared to the wild-type (Fig. 4.18). Since the alterations were observed so early in the chromatograms (15 min in 50 min HPLC program), it was unclear whether these results represent any important secondary metabolites.

AtMYB12 is highly expressed in young seedlings. Thus, we investigated whether DTX28 promoter will be active in the tissues of the seedlings of plants transformed with DTX28 promoter fused to β-glucoronidase. We detected that DTX28 promoter is active ubiquitously in the seedlings (Fig. 4.16), which might support the hypothesis of DTX28 being involved in flavonol transport. The subcellular localisation analyses indicate that *cDTX28-GFP* localizes to the plasma membrane, which confirms Cell eFP browser (http://www.bar.utoronto.ca/) prediction for that gene (Fig. 4.17). Further studies should verify the plasma membrane localisation of DTX28. In case that DTX28 is indeed localised in the plasma membrane, HPLC analysis of the apoplastic sap and root exudates may hold to identify the substrate of DTX28.

CHAPTER V: GENERAL DISCUSSION, CONCLUSIONS AND OUTLOOK

5.1. GENERAL DISCUSSION

Elucidation of the mechanisms underpinning flavonoid biosynthesis and regulation provides the toolbox to genetically engineer plants to introduce valuable nutritional and forage quality traits. To date, flavonoid biosynthesis is one of the best characterized metabolic pathways in plants. Yet, the mechanisms by which these phenolic compounds reach their cellular destination are not fully understood.

Recent studies implicate both ABC and MATE transporters in the transport of plant secondary metabolites, including flavonoids. However, biochemical data on transport properties and functional aspects of plant MATEs is still limited and no information is known of amino acid/s that are critical to the function and targeting capacity of plant MATEs.

In this work, we aimed to elucidate (i) functionally important amino acids of the plant MATE family using the TT12 MATE protein as a model and (ii) to identify novel MATE transporters involved in flavonoid transport. To this end we undertook an approach to identify amino acids critical to the transport activity of model TT12 by site-directed mutagenesis. Furthermore, we aimed to functionally characterize two putative MATE transporters from *Lupinus albus* previously identified in the group. These putative MATEs were suggested to function in the extrusion of isoflavonoids and citric acid in white lupin proteoid roots under phosphate starvation (Tomasi *et al*, 2008). Finally, using an Arabidopsis model we attempted to identify novel putative MATE transporters which could play a role in flavonoid transport.

The major findings of this work are summarised as follows.

We attempted to biochemically and functionally characterize putative MATE transporters in white lupins that were previously thought to be involved either in citrate or isoflavonoids excretion from proteoid roots under phosphate deficiency (Tomasi *et al*, 2008). We heterologously expressed the cDNAs of both lupin MATEs (*LaMATE1* and *-2*) in yeast and conducted transport experiments on intact microsomes to investigate their proposed transport activities. We carried out citrate uptake experiments for *LaMATE1*, which was predicted to be involved in the excretion of organic acids into the rhizosphere during phosphate starvation. We did not observe any transport activity in the presence of citrate for *LaMATE1*. Similarly, *LaMATE2* was suggested to excrete isoflavonoids under the same conditions. We demonstrated that yeast microsomes expressing *LaMATE2* were able to transport genistein, an isoflavonoid, which was previously shown to be secreted into the rhizosphere in response to phosphorus starvation (Weisskopf *et al*, 2006). This transport activity was dependent on

the presence of MgATP and therefore demonstrated that it may encode an isoflavonoid proton antiporter.

Our laboratory previously demonstrated explicitly that the Arabidopsis MATE *TT12* (At3g59030) was a glucosylated anthocyanin transporter functioning in the accumulation of PAs in the seed coat endothelium (Marinova *et al*, 2007). Since this gene was well characterised and the relevant mutation shown to have a striking PA deficient seed coat we decided to use TT12 as a convenient model to investigate which amino acids are crucial for proper functioning of plant MATE transporters. To date, the identification of conserved amino acid signatures or domains essential to the transport function remains poorly characterised, with no reports for plant MATEs. Amino acid residues critical for the transport function were characterized for NorM from *Vibrio parahaemolyticus* (Otsuka *et al*, 2005) and hMATE1 from *Homo sapiens* (Otsuka *et al*, 2005). Two crucial amino acids residues were demonstrated for those organisms to which we found homologous amino acid residue in TT12. The amino acid residue E290 together with 11 other amino acid residues conserved in Arabidopsis MATE family were mutated in the site-directed mutagenesis approach and a series of experiments were conducted. Specifically we looked for mutations in TT12 that were unable to functionally complement the PA deficient seeds of the *tt12* mutant background. We demonstrated that a single amino acid residue of TT12 (E290) failed to complement the *tt12* seed phenotype. When this mutated version TT12 was heterologously expressed in yeast, microsomes were unable to transport cyanidin-3-glucoside, whilst the native version of TT12 did. This result confirmed previous findings (Marinova *et al*, 2007) but also explicitly demonstrated that the E290 mutation completely abolished the transport capacity of TT12. This work presented the first evidence of the functional amino acid residues in plant MATE transporters, clearly revealing that a single amino acid residue is crucial to the transport function of TT12. Interestingly the Arabidopsis TT12 E290 is conserved in the model MATE transporters from *Vibrio parahaemolyticus* (NorM) (Otsuka *et al*, 2005) and *Homo sapiens* (hMATE1) (Otsuka *et al*, 2005). This residue was also shown to be critical to physiological activity in these MATEs and suggests that this may represent a universally conserved functional residue in MATE family.

Since only a few MATE transporters were characterised in Arabidopsis, we hypothesized that Arabidopsis MATEs phylogenetically related to TT12 could represent novel flavonoid transporters. Based on both *in silico* analyses and literature searches, we chose three MATEs thought to show a promise as putative flavonoid transporters (DTX28, 35 and 31). Using a classical reverse genetic approach we attempted to ascertain of gene loss-of-function which influences the flavonoid composition.

Firstly, since anthocyanins are reported to hyperaccumulate in Arabidopsis when the MYB75 (PAP1) transcription factor is constitutively overexpressed we used Ox-PAP1-D transgenic background to

investigate the effects of RNAi silencing of DTX35. We observed that PAP1-D lines super-transformed with a $DTX35_{RNAi}$ silencing construct exhibited a strong phenotype, no longer hyperaccumulating anthocyanins (red colour reverted to green). Unpredictably, semi-quantitative reverse transcription PCR (sqPCR) analyses of selected $DTX35_{RNAi}$ lines showing this phenotype reversion displayed reduced transcript abundance in key flavonoid biosynthetic genes (*PAL1*, *CHS* and *DFR*) suggesting that the expression of the $DTX35_{RNAi}$ silencing construct led to unpredicted attenuation of they key genes and that collectively this resulted in the phenotype reversion. Furthermore, our initial analyses focused on investigating physiological functions of *DTX35* in vegetative tissues (leaves and roots). However, a recent report identified *DTX35* as a flavonoid transporter required for anther dehiscence and pollen development (Thompson *et al*, 2010) with an expression pattern strongest in these organs. Given that DTX35 has been functionally and physiologically described, the *DTX35* project has been abandoned.

AtDTX28 was chosen since it was suggested that this gene was involved in flavonol allocation. It is strongly induced after UV-B treatment in Arabidopsis and this expression pattern positively correlates to an increase of expression of MYB12, a transcription factor involved in the regulation of flavonol biosynthesis. We began investigation using (i) HPLC-RID analyses of flavonol patterns in a T-DNA insertion line mutants compared to the wild-type plants; (ii) sqPCR to investigate *DTX28* transcript abundance in a transgenic Arabidopsis line overexpressing MYB12 and myb12 T-DNA insertion mutant and a triple knockout mutant *myb11-myb12-myb111* (all three TFs are known to regulate flavonol biosynthesis in Arabidopsis). Our findings did not support the hypothesis that *DTX28* may represent a putative flavonol transporter. HPLC-RID analyses did not reveal any significant differences in the flavonol pattern of the T-DNA insertion mutant. Moreover, the sqPCR experiments did not reveal any differences in *DTX28* transcript abundance in the backgrounds described above, indicating that DTX28 is not regulated by TFs involved in flavonol biosynthesis. However, in Arabidopsis the MATE family is constituted by 56 members and it may be possible that some MATEs have overlapping function. Therefore, we cannot exclude that AtDTX28 is not a flavonol transporter which is not co-regulated with the enzymes responsible for flavonol biosynthesis. Future experiments should verify the plasma membrane localisation of DTX28 and, in case of confirmation, aim to identify the substrate of DTX28 by HPLC analysis of root exudates and appoplastic sap.

Transcripts of *DTX31* were specifically upregulated in Arabidopsis roots only in response to salt stress and not during osmotic. We could correlate this expression pattern to the activity of the *DTX31* promoter. Using a β-glucoronidase reporter gene, we found the strongest GUS staining pattern in roots subjected to a salt stress. Following recent findings from our group where flavonoids were shown to influence the correct distribution of auxin in the root tip during gravi stimulus (Santelia *et al*, 2008), we tested if DTX31 could be indirectly involved in the regulation of auxin distribution via

flavonoid transport. Salt overly sensitive mutants exhibit classical agravitropism during high salinity. We conducted an experiment to investigate whether a dtx31 T-DNA insertion mutant would exhibit agravitropic root growth under salt stress and therefore confirm its function in the negative regulation of auxin transport in response to gravitropism. Consequently, we demonstrated that *DTX31* is not responsible for the transport of flavonoids into the root tip in the gravitropic response. After observing the transient expression of the DTX31 transcript in correlation with DTX31 promoter activity in the roots, we performed HPLC analyses of root exudates collected from hydroponically grown dtx31 knock-out mutant and the wild-type plants and observed significant alteration and differences in the metabolic profiles. This is presently being pursued as collaboration with Dr. Laurent Bigler (University of Zurich) with the objective of identifying the candidate compounds (HPLC-ESI-MS/MS). The physiological function of DTX31 remains unclear although we have demonstrated that this MATE localises to the tonoplast. We still believe that it may somehow function in the salt stress response in Arabidopsis roots. The appearance of at least one new compound in the root exudates of the loss-of-function mutant may give a clue on the substrate transported by AtDTX31. Therefore it is of central interest to pursue the metabolic profiling and the identification of this compound. This result could also provide new information on secondary compounds temporarily required for coping with salt stress. Additionally, the second dtx31 loss-of-function mutant line will be investigated to verify the authenticity of the results we obtained from the first line we examined.

Collectively, the approach we undertook has its merits despite difficulties in precisely identifying new MATE transporters involved in flavonoid transport. However, identification of flavonoid transporters is a critical step and may help to avoid toxic effects in engineering PA biosynthesis in leaves and increase food quality (Sharma and Dixon, 2005). Elucidation of flavonoid transport mechanisms could be also important as a tool to design plants or cell culture systems that produce health-promoting phenolic compounds (bio-pharming). Lastly, the identification of crucial amino acid residues in TT12 provides a groundwork for a better understanding of the transport mechanism for other members of the Arabidopsis MATE family.

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LIST OF ABBREVIATIONS

ACMA	9-amino-6-chloro-2-methoxyacridine
At	*Arabidopsis thaliana*
BAN	BANYLUS
BSA	bovine serum albumin
C3G	cyanidin 3-glucoside
cDNA	complementary DNA
CHS	chalcone synthase
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
DFR	dihydroflavonol reductase
DMACA	p-dimethylaminocinnamaldehyde
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DTX	detoxification
DW	dry weight
EDTA	ethylenediaminetetraacetic
FW	fresh weight
GFP	green fluorescent protein
HPLC-RID	High-performance Liquid Chromatography with Refractive Index Detector
kB	kilobase
LaMATE	*Lupinus albus* MATE
LB	Luria Broth
MATE	multidrug and toxic extrusion
MS	Murashige and Skoog
n.d.	not detected
NASC	Nottingham Arabidopsis Stock Centre

PAs	proanthocyanidins
PAL1	phenylalanine ammonia lyase 1
PAP1-D	production of anthocyanin pigment 1-dominant
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
PVP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
RNAi	RNA interference
RT	room temperature
sqRT-PCR	semi-quantitative reverse transcriptase-polymerase chain reaction
SDS	sodiumdodecylsulphate
T-DNA	transferred DNA
TF	transcription factor
TT12	transparent testa 12
v/v	volume to volume solution
X-Gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexamine salt
YPD	Yeast Peptone-Dextrose

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